

28 May 1981

Does creation deserve equal time?

Reports from Arkansas (of which there are not many) suggest that the creationists have established a strong bridgehead there (see *Nature* 21 May, p.179). Creation and Darwin are to have equal time in the school curriculum, but how this will be done has not been decided (and may never be, if the American Council for Civil Liberties manages to overturn the bill rushed through the Arkansas legislature in March). The creationists of Arkansas would therefore be heartened by the exhibition on the "Origin of Species" mounted by the British Museum (Natural History) in London to mark its centenary this week. The exhibition, to be reviewed by Professor Barry Cox next week, is splendidly designed, persuasively instructive and, with a few exceptions, philosophically unobjectionable. But the exceptions are significant and striking. At the entrance to the exhibition gallery are two panels, one of which accurately describes the origins of Darwin's theory of the "Origin of Species". The other panel, almost but not quite as prominent, says flatly that there are other ways of accounting for the diversity of species, among which "the theory of Creation" stands out. The doctrine of equal time, hitherto used for deciding which politicians should speak on television for how long during election campaigns, is clearly making headway. Should it?

What can creationists fairly claim of time in the school curriculum or of space in public exhibitions? To what extent should teachers and exhibition designers pretend agnosticism when explaining neo-Darwinian notions of evolution by natural selection because they know that many of those they are addressing sincerely hold conflicting beliefs? Superficially, these questions resemble that of whether Darwin's theory, as now elaborated, is a scientific theory in Sir Karl Popper's sense (see *Nature* 12 March, p. 75 and later *Correspondence*). Certainly there are connections. If Darwinism is untestable by Popper's criteria, and thus by his narrow definition metaphysical, and if Creationism inspired by religious belief is also (but more frankly) metaphysical, are not the two theories equipotent and thus deserving of equal time? These are the arguments that have enabled those who call themselves scientific creationists to make such headway in the past few years, in the United States in particular. It is also likely that many of those who urge that Darwinism is "only a theory" do so because it does not conform with their religious convictions. Yet the issue of equal time is an issue in its own right, and one that deserves a simple resolution.

The issue is not a simple extension into this century of the fierce rows about God and Darwinism that occupied some of the Victorians, especially in Britain. Then, with religion firmly entrenched and sometimes backed by the force of law, it was natural that people such as Huxley should have seized on Darwinism as a way of substantiating their long-standing disbelief. Now, with the shoe on the other foot — religion less widely practised and in most places disestablished — the argument is different and more subtle. The scientific community is not, as popular legend has it, monolithically irreligious even if card-carrying believers are proportionately fewer than in the general population. And the fact that science and religion can coexist is a sign that, behaviourally, science and religion are not always antithetical. People, it seems, can spend their weekdays in laboratories observing phenomena which they suppose are causally determined and Sundays on their knees, believing that some events are differently arranged. (Sects differ in their estimates of the importance of physically non-causal events, as evidenced by their differing assessments of the function of prayer

somewhere between the extremes of cathartic wish-fulfillment and putatively effective interruptions of causal processes.)

To many professional people, this awkward philosophical compromise is offensive, but mistakenly. It is not even a philosophically untenable position to suppose that the present world is well-determined, but that it was all set in train in, say, 4004 BC, the year that Bishop Ussher figured out that the world began. Continuing inspired non-causalism is harder to accommodate, but even that circle can be squared in the minds of many people, professional scientists included. But much of modern religion is drawn in such a way that inconsistency crops up less openly. In any case, in most fields of scientific enquiry, the supposed conflict between science and religion is entirely irrelevant. For the practice of science, what matters is that investigators should be unprejudiced in their expectations of the outcome of an experiment or a series of observations, and unbiased in their interpretation thereof. Even so, with the passage of time, once inexplicable phenomena have become susceptible to physically causal explanations, so that the number of unsolved problems behind which God might be lurking has shrunk. And there are some fields of enquiry in which some (not all) religious convictions are an impediment to scientific enquiry. Fundamentalists who become palaeoanthropologists have a hard life, as may devotees of astrology who work in stellar dynamics. For the rest, however, the scientific community has a duty to be as tolerant of religious conviction among its members as of, say, differing political convictions. For the most part, it is.

The trouble, in Arkansas and the British Museum (Natural History) — which should change its name — is that tolerance has gone too far. It is one thing to be tolerant of religious conviction and quite another to give religiously inspired hypotheses equal time with theories which are scientific in the sense that (correct or otherwise) they purport to relate to effects and causes which are themselves capable of being understood. Correspondence in the past few months has shown that it is possible to argue, within the convention that effects are related to identifiable causes, about the validity of neo-Darwinism. The theory of Creation is not, as the museum's placard suggests, one of the possible refinements of neo-Darwinism that may in due course emerge from more careful study of evolutionary data but a radical alternative which has the further property of not being a scientific theory: however stated, the theory of Creation relates effects to causes which are *ad hoc* and, by definition, not susceptible to understanding.

If the museum had sought to advertise to those who will be flocking to its new exhibition during the next few months that the "theory of evolution would be abandoned tomorrow if a better theory appeared" (*Nature* 12 March, p.75), it would have done better to refer as an alternative to the theory that Hoyle and Wickramasinghe have been advocating in the past few years, which entails the repeated replenishment of the surface of the Earth with life forms from elsewhere. That, at least, will be capable of verification in the years ahead. If the objective were merely to avoid offending the small proportion of fundamentalists among its religious visitors, it could have simply said that Darwin's theory is the most persuasive of the scientific theories of evolution so far, and acknowledge that some people do not look for a scientific explanation of everything. To refer to the theory of Creation as an alternative to Darwinism is, however, to fudge the issue. Creation is not a different scientific theory but an explanation of the diversity of species of a different kind. Science museums, and science teachers, have the task of



cultivating an understanding of what scientific explanations are. Naturally, if some among their audiences prefer supernatural explanations, toleration is in order. But not equal time.

Organizing change

Last week was formative, even traumatic, for the British government's arrangements for the sponsorship of technical innovation. It began with a report from the Advisory Council for Applied Research and Development on operations of the National Research Development Corporation, set up immediately after the Second World War as a means of exploiting inventions not taken up by private industry, and which has been for the past two decades the chief means of exploiting inventions arising within publicly supported laboratories, in universities and elsewhere. The week finished with public confirmation that the corporation is to be merged, as soon as possible, with the other and more controversial arm of the British government's patronage of industrial innovation — the National Enterprise Board. By all accounts, both organizations have been working closely together since the beginning of the year, when Sir Frederick Wood, then chairman of the corporation, was appointed chairman of the board as well. Now there is to be legislation to formalize a merger.

Politically, the rearrangement will have considerable advantages. The National Enterprise Board is, for the present British government, a curious hangover from the past. It is the survivor of the organization set up in 1965 by Sir Harold Wilson's Labour government, which is best remembered for having spent a great deal of public money persuading companies to merge with each other, sometimes successfully (as with General Electric Company), sometimes unsuccessfully (British Leyland). The present British government, ideologically opposed to public intervention in private industry, has nevertheless found it necessary to keep the rump of the old organization if only as a means of disposing of the public investments that have accumulated over the years. But the government (like its supporters) has never been easy with this inherited embarrassment. It will obviously be of some assistance if the board can appear to disappear, and the name being canvassed for the new organization — the British Technology Corporation — may help in that direction.

The proposed merger nevertheless makes sense. Each of the proposed partners needs strengthening. The National Enterprise Board, accustomed as it is to persuading bankers to act in unfamiliar ways, is short of a knowledge of what technology is about. The corporation, on the other hand, knows a lot about technology but is financially unadventurous, perhaps because of its over-zealous avoidance of borrowing from the Treasury to enlarge its operations. In principle, then, the impending merger should provide an organization more effective than the sum of its parts. The combined organization may become the elusive ideal after which British governments have striven for decades — a cost-effective way of using public money to encourage industrial innovation.

This is why all those concerned with the merger should read the report of the advisory council with close attention. It is possible that the problem of supporting innovation with public funds has been transformed since the corporation and the board were first conceived. One of the complaints that the council makes against the corporation is that it has in the past been too concerned that innovations coming its way should already be protected by patents. Somebody convinced that his new mousetrap will drive all other mousetraps from the market must first take out a patent and then, if his invention has not been snapped up by a mousetrap manufacturer, must ask the corporation to help with the cost of development. The trouble, the council properly says, is that in present circumstances many of the most interesting innovations consist not of patentable inventions but of know-how, "intellectual property". The complaint against the National Research

Development Corporation is indeed softened by a recognition that the corporation has recently been changing its ways, but plainly a merger with the board will help in this direction, if only because the board is more used to backing companies than patents.

Another of the council's complaints against the present arrangements bears on the relationship between the National Research Development Corporation and the universities and government laboratories. Hitherto, the corporation has had a right to first refusal of all innovations springing from the use of public funds. It has not always been imaginative in its dealings with innovators in these places. Rightly or wrongly, many in this constituency have a sense of grievance. The advisory council now suggests that this monopoly should be dispensed with, on the grounds that there should be more than one source of support for promising ideas. This argument is not accepted by the National Research Development Corporation (which claims already to have acted on the council's other recommendations). The reluctance is understandable but unjustifiable.

Too much TV?

The geosynchronous orbit is a scarce natural resource. There is only one orbit about the Earth in which objects will appear to be stationary from any point on the surface of the Earth. Obviously such a unique facility should be used sensibly. By good fortune, national ambitions to put satellites into this orbit are moderated by international negotiation and agreement within the International Telecommunications Union, originally established to see that terrestrial broadcasting systems did not interfere with each other. Aware of the technical possibility that geosynchronous satellites might be used for broadcasting television signals directly into people's homes (with the help of a small dish antenna), the union called an international conference in 1977 to carve up the geosynchronous orbit among its member states. The report of the British Home Office (which combines responsibility for the regulation of domestic broadcasting with that for such matters as the administration of British prisons) shows that the decisions then taken should be reconsidered.

The report (see page 277) is stimulated by the British Government's wish not to waste the five channels of television broadcasting doled out to in 1977. But this is an awkward time. The Home Office has just suffered the trauma of deciding that British television-watchers should be allowed a fourth channel, to be run under the supervision of the Independent Broadcasting Authority and financed from the profits of the commercial television companies who occupy one of the three existing channels of domestic television. Much of the Home Office's strategy on broadcasting in the past few months has been designed not to harm the profits of the commercial television companies, for that would jeopardize the "success" of the fourth channel. Only this can explain the caution with which would-be operators of cable networks are being licensed. The offer of a further five television channels is not a gift but an embarrassment.

The British Government deserves some sympathy in its embarrassment. The 1977 decision that each member of the International Telecommunications Union should be entitled to five geosynchronous direct-broadcasting channels was ill-considered and premature. Then, as now, it was not clear what balance that will eventually be struck between direct broadcasting to people's homes and the more efficient use of the geosynchronous orbit by means of broadcasts to community antennas. The American members of the union had the sense to opt out of the 1977 decision, and will consider how the geosynchronous orbit should be carved up among themselves only in 1983. Elsewhere, the mere availability of the new channels is certain to lead to a waste of effort in the development of a broadcasting system whose economic place in the market cannot be determined while national governments (the British included) decline to let competing and complementary systems, such as cable networks, develop freely.

Budget cuts cast shadows overseas

US agencies count costs of Reagan

Washington

The Reagan Administration, stung by criticism from Western allies of budget-based decisions to terminate or substantially withdraw from a number of international scientific projects, is taking a close look at ways in which it may be able to repair some of the damage and prevent further unnecessary friction in the future.

Of particular concern to the new Administration is that the foreign policy function of certain types of international scientific agreements — for example, a science and technology programme agreed with Spain in 1976 as part of an exchange for being allowed to place US military bases in the country — could be jeopardized if the projects are evaluated merely on the strength of their scientific merits and the vocal energy of their domestic constituency, often very small within the scientific community.

Already, Secretary of State Alexander Haig has written to Mr Reagan's budget director, David Stockman, complaining of the fact that cuts imposed either directly by the Office of Management and Budget (OMB), or indirectly by internal decision within agencies such as the Department of Energy or the National Science Foundation (NSF), have been carried out in a manner which constitutes a unilateral abrogation of international commitments.

Among the projects listed by Mr Haig which appear to have been treated in this way is the international solar polar mission, planned jointly with the European Space Agency, from which the National Aeronautics and Space Administration (NASA) has proposed withdrawing its planned spacecraft (a decision which both congressional committees and NASA officials are busy trying to reverse, perhaps through a reduced NASA commitment which would involve the European Space Agency building the two spacecraft involved). Another is the possible cancellation of national energy assessments supported by the Department of Energy and already promised by embassies in countries such as Greece, Tunisia and Venezuela.

What concerns the State Department most is that the scientific attachés of foreign embassies in Washington are telling their capitals that US promises of scientific and technical collaboration should be looked at sceptically in the future. "While budget reductions are a clear goal for this Administration, one of its principal foreign policy objectives is to render the

United States a reliable international partner," wrote Mr Haig — who had previously interceded with OMB to rescue foreign aid funds and NASA's Galileo mission to Jupiter, planned with heavy West German involvement — in his letter to Mr Stockman.

International collaboration in scientific programmes is seen by Washington science policy officials as falling into three categories: that carried out by individual scientists and their institutions as part of the normal process of science; that in which both sides receive the benefit of more cost-effective technical knowledge; and that carried out with some broader foreign policy goal in mind.

It is the last of these three which is particularly threatened, particularly when an international treaty or bilateral agreement has been arranged through the State Department, and then handed to another agency for execution.

One major dilemma now facing the Reagan Administration, for example, is what to do about the International Institute for Applied Systems Analysis (IIASA) in Vienna. Faced with the need to make cuts of over \$300 million — almost 25 per cent — in its proposed 1982 budget by the new Administration, NSF decided to eliminate the \$3 million US contribution to IIASA for which it is at present responsible.

Unless this decision is changed by the Administration, IIASA would have to close in 1983. Scientists claim that the institute has been carrying out some useful research, even though some express doubts about its full value. However, IIASA also

provides an important channel for East/West communication.

Moves are therefore under way to discover whether the closure of the institute — which would anger the Austrian government considerably, since it has put a lot of money into providing facilities — can be averted. For the 1982 contribution, already committed under IIASA's constitution, NSF is working out whether it can provide money from other sources within the foundation.

Recognizing that the problems raised by the controversy over the international solar polar mission and IIASA contributions have deep roots in beliefs about the proper political role of the federal government in support for science, the State Department has set up an inter-agency committee to discuss possible guidelines for the future. Two suggestions it is likely to discuss are that OMB should be presented, early in its budget cycle, with an overall picture of overseas implications. The other is that the State Department itself might be given funds for supporting international scientific activities which it feels have important political functions, but might not generate the required support within an individual agency.

At the same time the agencies themselves are looking closely at their own policies and procedures. Few solutions are in sight, but one consolation to US scientists is that, as a result of recent events, the whole issue has been placed high on the State Department's agenda at an early stage and is already receiving close attention from what are usually described as the "top levels" of government.

David Dickson

Anxieties of Oslo secrets trial

Stockholm

A trial whose outcome could affect freedom of research is being held in Oslo. Two defendants are at present accused of revealing information prejudicial to Norway's security. But this is not a normal spy case: the defendants collected the information exclusively from public sources as part of a research project funded by the Norwegian Research Council for Science and the Humanities, and there is no suggestion that they intended to pass it on to any foreign power.

The two men are Nils Petter Gleditsch of the Oslo International Peace Research Institute (PRIO) and Owen Wilkes, a New Zealander formerly at PRIO and now at the Stockholm International Peace Research Institute. They maintain that freedom of research should be upheld, and that they have not in any case revealed information which could damage Norway's security. They say that what they have discovered from open sources, a foreign power (that is, the Soviet Union) could investigate in far more detail with

intelligence satellites. They conclude that the Norwegian government's reticence about military affairs is keeping secrets from the Norwegians themselves, not from the Russians.

The history of the case is this: Gleditsch and Wilkes published their research report, "Intelligence installations in Norway: their number, location, function and legality" in February 1979 as part of a PRIO project (still continuing) on the location and functions of military facilities in Norway. They timed the publication to coincide with another trial then being held, in which three Norwegians — a publishing house executive and two journalists who had collected the names of Norwegian secret servicemen — were accused of collecting information which could damage the country's national security. Gleditsch and Wilkes wanted to show how easy it is to obtain from open sources information considered to be very sensitive.

The prosecutor general ordered an investigation into the Gleditsch-Wilkes report, and in March 1979, the chief of

defence staff, General Sverre Hamre, told the Norwegian police that he considered it a threat to the activities of Norwegian defence and national security. Having questioned the authors and PRIO staff closely associated with preparing the report, the police completed their investigations in June 1979 and sent preliminary charges against Gleditsch to the court. They charged him on three counts: two under the penal code — revealing information which ought to be kept secret in the interests of national security relative to foreign powers, and acquiring this information or making it available to others — and one under the law on defence secrets — recording, copying, or publishing sketches of fortifications or associated installations. According to Norwegian practice, the police also appointed three independent experts to evaluate the report. The intelligence service proposed two of these (one was the former head of the service) and Gleditsch and Wilkes the third. Not surprisingly, the experts nominated by the intelligence service supported General Hamre's opinion that the report threatened national security. The formal indictment charges Wilkes as well as Gleditsch with all three charges, which carry a maximum penalty of 4½ years imprisonment.

The technical intelligence facilities described in the Gleditsch-Wilkes report collect and analyse data from electronic intelligence satellites, monitor and analyse military and diplomatic radio signals, and intercept and interpret electronic and tele-metric communications. Although the authorities are traditionally tight-lipped about such installations, the authors point out that it was very easy to find out about them by using, for example, the ordinary telephone directory and public lists of government-owned property. They also learned a lot by observing the installation's antennas: their size, shape and layout. The authors maintain that if the Soviet Union, with its sophisticated spy satellites and, presumably, other more traditional techniques of spying, is interested in the installations, it would already know a great deal about Norwegian security. They claim that the authorities' secrecy is mainly directed towards the Norwegian population itself, pointing to police reaction to the publication of their report in Norwegian, shortly before the trial began. For two years the report has been available in English and the police have made no attempt to confiscate it; however, they have now declared the publication of the Norwegian translation illegal.

Support for Gleditsch and Wilkes has come from the International Peace Research Association, the Norwegian Writers' Union and political youth organizations in Norway. The trial is expected to last for another week or so, and the verdict will follow a couple of weeks after that. One or both sides will almost certainly appeal.

Wendy Barnaby

UK nuclear power

Plans panned

For the second time this year, Britain's Central Electricity Generating Board (CEGB) has received a rap over the knuckles for not providing enough information about the economic case for its planned nuclear power programme. Hard on the heels of sharp criticism from the House of Commons Select Committee on Energy last March, the Monopolies and Mergers Commission last week published a report on the board's finances (HC 315, HMSO, £9.30). The Monopolies Commission was asked to find out whether CEGB is charging a fair price for the electricity which it supplies to the consumer through regional electricity boards.

The commission says that one of the chief reasons for the high cost of electricity in Britain is CEGB's previous tendency to invest in new plant before strictly necessary. Decisions to order new plant, it concluded, have often been based on over-

estimates of future demand, and economic cases have been made on over-optimistic estimates of construction times, capital costs and the ultimate performance of power stations.

The commission's report is particularly critical of the decision, to build the second advanced gas-cooled reactor at Heysham, taken in 1979 as part of the government's decision to embark on a further nuclear programme, probably based on the Westinghouse design of the pressurized water reactor. Failing approval at next year's public inquiry, CEGB hopes to keep open the option of basing the programme on the notoriously costly British-designed AGR by building Heysham II.

Although the commission acknowledges that CEGB has learnt from its previous mistakes with the AGR, it still criticizes the board for not providing sufficiently detailed cost estimates for Heysham II, even though the decision to build it was taken largely on strategic grounds.

The commission also faults CEGB's case for future nuclear power stations on economic grounds. It says that the board's estimate of net effective cost assumes unrealistic improvements in future construction time and operational performance. Margins of error in cost estimates, together with a better indication of the board's assumptions, should have been presented to the government when making an economic case for the nuclear programme. The board should also have compared the cost of building new nuclear stations with the cost of refurbishing old coal-fired stations.

The commission, however, has praised the way in which CEGB keeps a check on its current expenditure and seems to be impressed with the performance of its Barnwood division, unpopular with many of its contractors, in keeping a detailed check on cost increases during construction. The commission's report, however, ends on a rather gloomy note. Even if CEGB implements all its recommendations, the commission cannot foresee any substantial cut in electricity prices to the consumer.

Judy Redfearn

Gloom on research

A high-level meeting of research councils from Germany, France, the United Kingdom and the United States was held in the English country town of Abingdon on the weekend of 16–17 May. The occasion, arranged by Sir Geoffrey Allen, chairman of the British Science and Engineering Research Council, is the second in a series of consultations begun with a meeting called by Professor H. R. Leibnitz, then the president of the Deutsche Forschung Gemeinschaft in June last year.

The objective appears to have been an exchange of views, most of them gloomy. The United States participants (Dr John Slaughter and Dr H. R. Langenberg, respectively director and deputy director of the National Science Foundation) left their fellow-administrators with the impression that the Reagan budget had been a body blow whose effects will not be confined to the United States, but that the United States is also worried by more long-standing problems — the difficulty of recruiting teachers of engineering for United States universities, for example.

European participants in the meeting are said to have welcomed their transatlantic colleagues into the company of the impoverished, and to have urged that if the United States now faces a period of entrenchment it would be worthwhile thinking of collaboration with European collaborative ventures such as those at CERN. The occasion seems also to have been one for concerted European complaint about the budgetary threat to the solar polar mission, the plan to send two spacecraft (one European, one American) into polar orbits about the Sun.

US science funding

Rewards of genius

Washington

Acting on the theory that intellectual and cultural breakthroughs can be accelerated if latent genius is permitted to flourish free of the more material considerations of making a daily living, one of the newest — and wealthiest — foundations in America has announced the first of a series of five-year, no-strings-attached awards to "exceptionally talented" individuals.

The awards are being made by the trustees of the John D. and Catherine MacArthur Foundation set up two years ago from the estate of the late Chicago insurance and real estate millionaire. The

foundation, which has assets of \$841 million, last week announced 21 individual awards worth a total of over \$4 million, with another 29 expected later in the year.

Eight of the initial recipients are scientists, and include Stephen Wolfram, a 21-year-old physicist at the California Institute of Technology, the Harvard geologist and paleontologist Stephen Jay Gould, and oceanographer and climatologist John Imbrie of Brown University and the University of Rhode Island.

The names were chosen by the trustees of the foundation from a list of nominations proposed by 100 educators, scientists and artists who acted as scouts in what other foundations, more conventional in their granting of awards, have dubbed the "search for genius". There are no conditions attached to the way that the money — between \$24,000 and \$60,000 a year for each individual, depending solely on age — can be used, nor can the award be withdrawn within the five-year period.

The MacArthur Foundation's novel approach to the support of intellectual activities is perhaps the most ambitious of a number of attempts to meet the charge that more conventional forms of funding discourage innovative or risk-taking work.

Last year, for example, Berkeley physicist Richard A. Muller, winner of the National Science Foundation's (NSF) Alan T. Waterman award based on research for which he had initially encountered difficulty in obtaining support, told a congressional committee that individuals engaged in innovative research often had similar experiences, for example when their proposed project did not fit neatly into one or another disciplinary compartment.

Dr Muller's testimony and other similar complaints led Congress to ask NSF to assess current funding mechanisms to find out how well they are working. So far these studies have not uncovered any substantial problems, nor pointed to any particularly radical solutions.

Furthermore, a task group set up last year by NSF's advisory council on the funding of innovative high risk proposals has reported that "on the whole, the foundation's procedures seem to be effective".

In the light of the comments received and of its own investigations, the task force, headed by Halsey Royden, dean of the school of humanities and sciences at Stanford University, suggested that NSF programme officers be given greater encouragement to support innovative risk-taking proposals and that a small Group on Innovative Research Topics be set up under the deputy director, to "promote promising research that does not fit naturally into the framework of existing programs and divisions of the NSF."

Both proposals are now being considered by NSF. However, Dr Langenberg points out that the task force specific recommendations are likely to be absorbed into the bigger organizational changes now under way.

David Dickson

High-energy physics

On the rocks

The Swiss tunnel expert Giovanni Lombardi, who has honeycombed the Alps with road and rail tunnels, denied last week that it might be impossible to build the tunnel for LEP — the next big project of the European nuclear physics laboratory CERN.

The assertion, in the British magazine *Consulting Engineer*, would have prevented governments from approving LEP construction at next month's crucial CERN Council meeting. Lombardi is not only a world-recognized expert on Alpine tunnelling but also CERN's principal geological adviser.

Lombardi and CERN do however admit to geological difficulties in that part of the LEP tunnel which will go under the limestone of the Jura, to the north-east of the CERN site. The worst of the troubles have however been avoided by shrinking LEP from 30 km to 27 km circumference, it is said. In this way, the tunnel should avoid the folded core of the Jura, a region of unstable limestone which Lombardi knows to contain water and mud-filled caverns.

Even so, progress through the Jura will be uncertain and based on "forage à l'avancement", where a small (2-inch) hole is drilled 20–30 m ahead of the main borer to probe for boundaries between limestone layers. At hundreds of metres below the water table, there may be mud and pebble-filled "karsts" at these rock divisions which will have to be emptied and filled with concrete before drilling through. If water-flow through the karst is too great, it can be impossible to place the concrete.

Uncertainties of this kind have persuaded smaller member states of CERN, led by Sweden, to demand guarantees that the CERN budget will not be raised to meet any extra costs. It has thus been agreed that future CERN budget increases can be vetoed by any state, while for decreases a two-thirds majority is sufficient. And CERN's director-general, Herwig Schopper, has agreed in principle that LEP cost escalations would be met by lengthening the time over which LEP is built.

Strenuous efforts to delineate the geology of the Jura are under way at CERN, but the principal reconnaissance gallery will not reach the tricky region until April next year. So CERN is also drilling a hole vertically above the deepest part of the proposed tunnel under the Jura and making geophysical observations from within it to find the water table and the trend lines of the various limestone boundaries. The results of this investigation will not, however, be known for another three months.

Meanwhile CERN is preparing for its mid-year council meeting on 25 June at which delegations from the 12 member states would normally approve the 1982

budget. This year the budget contains an appropriation for LEP, which is not being costed separately. The debate will centre on precisely what level the budget should take, and what guarantees can be given on LEP cost overruns.

Sweden, apart from its doubts on the latter score, is in political crisis, and will almost certainly abstain; Norway may do the same; and the Netherlands are in the midst of elections and cannot predict their position. Moreover, the CERN Council delegations of many of the member states have not yet been officially briefed (this includes Britain) and so the outcome is far from certain. Procedurally, if eight states vote for the budget including LEP, and none votes against, LEP can go ahead, and this seems likely, although Schopper would like to leave the door open for a few months after June to achieve a unanimous decision.

Robert Walgate

Hormone legislation

Consumer protest

Brussels

The failure of the European Community's council of agriculture ministers to make significant progress on banning the use of natural and artificial hormones in livestock production is forcing European consumers to take retaliatory measures. The Bureau of European Consumers' Association is now trying to persuade the sympathetic member states to block meat imports from the United States, New Zealand, Australia and elsewhere.

Last September, the Community agreed in principle to ban the use of all hormones in livestock breeding. The decision was hailed as a victory for the consumers but has since proved to be a hollow one. It has been suggested that the September council failed to understand the difference between natural and artificial hormones and hence the problems of forbidding the use of the former. On 12 May, the agriculture ministers met to consider the European Commission's two proposals for directives to implement the ban — the outcome was disappointing.

A German proposal was adopted banning some artificial hormones already forbidden under existing laws operating in all member states except the United Kingdom. Diethylstilboestrol and other stilbenes are now to be banned, although whether this entails a separate directive or merely the partial implementation of the Commission's all-embracing directive is unclear. The other growth hormones, and the problem of enforcing any bans, will again be considered by the next agriculture council on 15 June.

The United Kingdom is becoming increasingly isolated in the discussions. The philosophy of not to ban a hormone until it has proved to be dangerous resembles that of the United States, but the legislation of other Community countries reveals a much

more cautious attitude. The United Kingdom has, however, been able to persuade its partners not to adopt the wide-sweeping legislation the Commission proposes until further studies have been carried out.

Various scientific committees will now be given the job of examining the health hazards of natural oestradiol, testosterone, progesterone, trenbolone (a processed natural hormone), zeranol and perhaps others. The United Kingdom rules permit the use of these hormones, which many other member states have forbidden. Similarly, until there is "conclusive" scientific evidence to the contrary, oestrogens, androgens and gestagens (except stilbenes) are considered safe, as far as the Community is concerned, for therapeutic use and to regulate the menstrual cycle.

The consumers feel that this scientific research could drag on for a long time, and matters will not be helped by the United Kingdom taking over the council presidency after the summer from the Dutch, who support tough Community legislation.

Jasper Becker

Canadian radioastronomy

Longer bases

Washington

Canadian astronomers have started design work on what it is hoped will eventually be the largest radioastronomy facility in the world. An eight-dish array of 32-metre antennas is to be distributed along a 5,000-kilometre line stretching from one end of the country to the other.

Canada's National Research Council (NRC) has already given its formal approval to the project, provisionally named CASCADE (after the Canadian Astronomy Society). Meeting in Ottawa in February, the council members gave the array top priority over four other possible national facilities that had been suggested for funding: a high-energy electron ring, an orbiting observatory, a kaon antiproton physics facility, and the creation of several centres of acoustics.

NRC and the Natural Sciences and Engineering Research Council have now agreed to share the costs of a \$300,000 design study for the array being carried out by a technical committee chaired by Dr Ernest Seaquist of the University of Toronto's department of astronomy. It is hoped that detailed plans for the array, estimated to cost about \$30 million at 1981 prices, can be completed by the beginning of next year; and that if the Canadian government can then be persuaded to support the project, funding for construction be provided in 1983.

Plans for a very long base array — which will be able to provide an angular resolution of 5×10^{-4} arc seconds at a wavelength of 1.5 centimetres, a hundred times better than can at present be provided by

the largest Earth-based radiotelescope — have been discussed for the past three years within the Canadian astronomy community, one of the first to develop and work with very long baseline interferometry. Two years ago, the very long base array came out on top of two other proposals, a 100-metre centimetre-wavelength dish and a large (25–30 metre) millimetre-wavelength antenna, in a study conducted by the Canadian Astronomy Society.

Despite enthusiastic support from astronomers, there was initially some scepticism from industrialists in NRC, who doubted that the project would have sufficient economic pay-off to justify the initial capital investment. However, after considerable lobbying several companies, particularly in the electronics, computing and telecommunications industries, were persuaded to back the proposal, particularly after it was pointed out that most of the components would be built in Canada. Astronomers argued, for example, that the increased production of antennas and equipment, together with some improvement in high frequency performance, would help Canadian aerospace and communications companies to compete for foreign markets.

As planned, the array would stretch along an east-west line from Newfoundland to Vancouver Island. Signals would be recorded independently at each station and subsequently correlated at a central processing facility, giving 28 components of the Fourier transform of the image of a cosmic radio source.

Efforts are being made to generate public support for the array by appealing to nationalistic instincts. A circular distributed by the chairman of NRC, Dr Larkin Kirwan, says the array would "assure Canadian leadership in radioastronomy for at least thirty years", and the Canadian Astronomy Society says it would "remain a permanent advertisement for Canadian science and technology".

In a similar vein, the secretary of the design committee, Dr Brian Andrew of NRC's Herzberg Institute of Astrophysics, heads a public relations committee whose task he describes as being "to elevate the array to the status of a national shrine." He also points out that the planned array would be able to take advantage of Canada's unique geography, and that "linking the country from end to end seems to have intangible overtones that would make it politically attractive".

Meanwhile budget restrictions have forced US scientists to put back their own plans, developed last year by a group at the California Institute of Technology and its Jet Propulsion Laboratory, for a two-dimensional array stretching across the continent of the United States, and including antennas on both Hawaii and Alaska (*Nature* 288, 4; 1980).

Similar in size, conception and cost to the Canadian plans, the US proposal is said

to have been given top priority for the next decade by the Field committee now preparing a report on the future of US astronomy for the National Academy of Sciences. A north-south array would fit well with Canada's plan.

However, one of the victims of the budget cuts announced by President Ronald Reagan in March was a 25-metre millimetre-wavelength radiotelescope planned for Mauna Kea in Hawaii, and previously approved for funding through the National Science Foundation by the Carter Administration. With this project placed back in the melting pot it seems unlikely that other new capital construction will get much consideration in the next few years.

David Dickson

Hungarian agriculture

Economic growth

Hungarian agriculture is to be remodelled in the next twenty years on ecological principles. This is the burden of a report last month to the Academy of Sciences of an interdisciplinary survey of the country's "agroecological potential". The survey was first proposed at the 1978 annual general meeting of the academy by Dr Istvan Lang, at that time deputy general secretary. Thirty research institutes, universities and computer centres took part, and more than 400 scientists were involved.

The survey asked three main questions: what quantity of agricultural plant production can realistically be attained by the end of the century? What conclusions can be made about the long-term targets of economic policy? How can production be increased and costs reduced in the medium term? The preliminary conclusion is that in the most favourable conditions, the annual grain yield could reach 22 million tonnes (present level 12–13 million tonnes) and that the productivity of grasslands could be doubled.

Nevertheless, the survey notes, there are constraints on the development of Hungarian agriculture. The country is poor in fossil fuels, so that with rising oil prices a point may be reached where it is economic to settle for lower than maximum yields and less expenditure on fertilizers. The country's geology is also a constraint. Hungary lies in the lowest part of the Carpathian basin, and the run-off from the mountains has produced tracts of saline and/or alkaline soils, principally in the western half of the country. Thus the Puszta remains a virtual desert in spite of an annual precipitation of 550 mm. Yet normal leaching methods of reclamation are ineffectual, since the soil is so impermeable that the water would simply pond on the surface. Moreover, Hungary has no means of disposing of the drain water, since the quality of the rivers Tisza and Danube must be ensured at the southern frontier.

Nevertheless, on the Comecon scale of

"relative climatic productivity", which assigns an index of 100 to the Soviet Union south of latitude 60°, Hungary scores 139, second only to Bulgaria with 145, and agricultural products, especially fruit and vegetables, account for some 30 per cent of Hungary's export earnings.

The goal of relating agriculture more closely to ecology was accepted in the five-year plan approved last year. The Hungarian economy has, however, come a long way from the central planning of the post-war Rakosi regime which, in an attempt to make the country self-sufficient, ordered certain collective farms to grow cotton and citrus fruits — with predictably poor results. Since the 1968 economic reform, the managements of the various collective farms have complete autonomy in deciding what they should grow. The only constraints are now those imposed by market prices and government incentives.

Often the consequences have been nonsensical. In the great sugar-beet affair of the mid-1970s, farmers applied more fertilizer than recommended and produced gigantic beet with no increase in sugar content. (Bonuses are now related to sugar yield, not gross weight.) In a more recent scandal, farms have obtained land improvement grants for top-soil dressing and then applied the soil to areas which were already of fairly high quality. This produced money for the farms, and excellent statistics for the soil improvement service — but a net loss to the economy.

The next step towards ecologically orientated agriculture will depend on the working out of suitable incentives for collective farms — free, it is hoped, from the loopholes and misunderstandings of the past.

Vera Rich

Direct satellite broadcasting

More TV ahead

Britain could start direct broadcasting by satellite in the mid-1980s. That is the government's response to the report of a Home Office study commissioned a year ago and published last week. The government favours a modest start in about 1986 beginning with two television channels and possibly some information services.

No change in the way broadcasting is controlled is, however, proposed, nor will the British government, unlike its French and German counterparts, help finance the venture. It hopes that British industry and the broadcasting authorities will be able to raise the money, estimated by the report to be £75–£160 million of capital to establish a system, depending on the type of satellite and the number of channels chosen. Throughout a 10-year period, the annual cost of operating a channel, including original capital costs, is estimated at £10–16 million. In addition, the broadcasting authorities would need to spend between £10 and £100 million a year

to run the service for 50 hours a week.

The Home Office study was stimulated by the fear that delay could put British industry at a disadvantage in the supply of equipment to what is expected to be a rapidly growing world market. The Home Office also had to work out a way of using the allocation in 1977 of five television channels from geostationary satellites.

There are two possible systems to choose from — a modified version of the European Communications Satellite and a satellite based on L-sat, the prototype of the second generation of telecommunications satellites due for approval by the European Space Agency next month. Signals could be beamed direct to individual 2–3-m dishes or to larger community antennas. Control of programmes would remain with the British Broadcasting Corporation (BBC) and Independent Broadcasting Authority (IBA), which control all British broadcasting through terrestrial systems, although coverage would extend to neighbouring countries.

The investigation uncovered a mixed response to direct broadcasting. The aerospace and electronics manufacturers are the most enthusiastic. The BBC is also keen, believing that it could use two channels — one to transmit repeats of programmes already shown on terrestrial channels and paid for from the standard television licence fee, and the other to transmit new special programmes, paid for by subscription. But the IBA, grappling with the introduction of breakfast television and a fourth television channel, does not think there will be a market before 1990.

The technical problems are less forbidding than the financial. The broadcasting authorities, which do not have £75–£160 million to invest, would prefer to lease channels from an operator. Private industry, especially the newly-formed Satellite Broadcasting Company Limited, has expressed some interest in launching a satellite and operating it as a common carrier, but industry's willingness to invest will depend on its confidence that a market can be found.

The option of using L-sat to test the British market has been preempted by the Italian broadcasting authorities, which are planning to run an experimental project.

Elsewhere in Europe, France and Germany are planning to take up their allocation of five channels by means of joint ventures. Two of the French channels will be used for broadcasting signals now transmitted terrestrially. The prospect that a third channel might be leased to Luxembourg has faded, while plans for an internationally financed Swiss satellite, aimed at providing commercial television programmes for the whole of Europe, have been set back by the rules on foreign investment in Switzerland. Similarly, the Scandinavian plan for a regional broadcasting service by satellite is in limbo.

Judy Redfearn

Cooling sackcloth

New Delhi

This year Indians are beating the summer heat by installing a novel air conditioner on the terraces of homes and offices. They spread a number of empty, used gunny bags (sack made of jute) over the terrace and keep them soaked with water round the clock with the help of sprayers like those used on lawns. This simple system works like an air conditioner. The Sun's heat evaporates the water instead of warming up the roof, and the process of evaporation cools the roof. In other words, the water-soaked gunny bags reverse the heat flow — and the heat from the house is thrown outside through the roof instead of the other way.

A fall of as much as 10°C in indoor temperatures has been observed during day times with this technique. Sleeping conditions at night are much better as the ceiling fans, a common fixture in most homes, throw out cold air because the roof is even cooler than the floor.

The "gunny bag air conditioner" has been developed by the Central Building Research Institute (CBRI), who say it is a cheap alternative to the desert coolers and air conditioners that only a few Indians can afford, and which consume electricity that is in short supply. The cost of installing the system per square metre of roof surface is 5.50 rupees (£0.30) and the water requirement is 9 litres per day.

CBRI started experimenting with its technique on its own buildings at the Roorkee campus. Results of these experiments, carried out during three successive summers, were so good that several factories and offices became interested. The system was installed on a 1,650 square metre roof of the four-storey building of Bharat Heavy Electricals Ltd in Hardwar. After the gunny bag treatment, the roof temperature of the building fell from 45°C to 28°C and indoor temperature from 39°C to 30°C. According to CBRI scientists S. P. Jain and Vinod Kumar, "not only the top floor but also rooms in the down floor were cooled as a result of constant working of the system".

CBRI engineers say that passive cooling by roof surface evaporation is suitable not only for India but for all developing countries in the tropics where artificial air conditioning is not within the reach of common man.

So far the roof surface evaporative cooling system has proved successful only for buildings with flat roofs. Attempts to use the system in roofs that are not flat are under way, and there are plans to develop inorganic water retentive materials as a substitute for gunny bags, which need replacement every three years or so.

K.S. Jayaraman

CORRESPONDENCE

Future reference

SIR — The recent letter from Andrew Brooks (*Nature* 7 May, p.7) suggesting that research students should take a course in information sciences recalls Dr Johnson's comment "Knowledge is of two kinds. We know a subject ourselves, or we know where we can find information about it". The combination of a continued growth in scientific literature and the worldwide cutback in library budgets is making it more difficult for us to "know where we can find information about it".

The long-term solution appears to be electronic publishing; a range of databases giving bibliographic and abstract information backed up by huge databanks where the original text is stored in machine-readable form for full retrieval. It has been estimated that in 1979 there were in the United States 93 databases in agriculture, life sciences, pure and applied science, producing 26 million records, while in Europe there are 92 databases in the same subject area producing 19 million records.

Commentators predict that these databases will grow rapidly over the next five years. Users will no longer expect to find the article they require in their library but with the aid of on-line access to the databases will be able rapidly to identify the literature relevant to their needs and to call it up from the databanks. Time spent on training should be more than compensated for by savings in time spent on the customary literature search.

ROBERT CAMPBELL

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Badger strain

SIR — In your correspondence about the Zuckerman Report, little has been said about the more medical aspects of the problem.

The report opens by confusing the issue. In the first paragraph the disease "tuberculosis" is referred to and figures given for its incidence in the United Kingdom. However, tuberculosis is a term covering the disease state produced by infections with more than one strain (some would argue more than one species) of bacterium. Man can be infected with *humanis*, *bovis* and *avium* strains. It is only strain *bovis* that concerns us in badgers. Paragraph 82 does rather belatedly clarify this, and implies that the figures on the first page overestimate the human risk by sixty-fold.

There is an even more dubious presentation of data in paragraph 58, where it is stated that the "bovine strain was four times more responsible for abdominal tuberculosis" in children (my emphasis). These figures are from a survey between the wars, when abdominal tuberculosis was commonly contracted by ingesting infected milk, while other forms (especially pulmonary) were transmitted more by human contact. Hence, *bovis* predominated in abdominal cases (before most milk was pasteurized), and *Humanis* in many others. (Abdominal tuberculosis used also to be common in cats, another group, besides children, that ingested large quantities of raw milk!) I fail to see why data were selected for cases in children, and

for this particular anatomical site, without some explanation.

The report goes to great lengths to destroy various falsehoods that have been in circulation. The above two examples refer to the cases respectively that there is a real threat to man, and that *bovis* does infect man. The fact that they in my opinion overstate the case in the support of what is in basis a sound argument does much to undermine the authority and objective position of the report.

It is also disappointing that the chapter dealing with the measures that have been taken is so small a part of the report. Here again I think a full picture is not presented.

The report also states (paragraph 107) that vaccination is not allowed for livestock. What it does not state is that in no way can this affect the question of badger vaccination — vaccination of cattle would interfere with the tuberculin test, but the test is of no use in badgers. (Curiously, the fact that the test does not work in badgers is then used as a reason against vaccination.) No explanation is given as to why infected badgers should not be vaccinated (as would happen unwittingly if blanket vaccination were practised). There are possible objections to vaccinating infected animals, but whether these would be outweighed by the advantages of blanket vaccination is an open question.

The report does not raise the question of the effectiveness of BCG in badgers. If it is not known, I would have thought it a prime research aim. If some of the badgers could be protected for some time, this would reduce the susceptible population.

Further, long-term policy is not well explored. The report supports the ministry's policy, but does not state what effect is expected. Is the policy to eradicate the disease or just to control it? Present measures seem to be only controlling it — is not that in itself a justification for a rethink on policy?

I am convinced of the sincerity of the ministry's actions, but I am also convinced of the sincerity of many zoologists who doubt the basis of those policies. Unless those doubts are considered I do not see how there can be public confidence in the ministry's policies. The association between state veterinary medicine and wildlife epidemiology is young, but of utmost importance as long as rabies lingers on the other side of the English Channel. I for one would welcome a widely based inquiry into ministry policy on both the current problem and those plans we hope we will never need.

M.J. CHAPMAN

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Paradigm change

SIR — It is now well understood that the abandonment of one time-honoured theory or paradigm in favour of another by scientists does not solely depend on proof¹. It also takes a substantial time for the whole scientific community to accomplish such a paradigm change². But for individual scientists the time taken can be much shorter. I have personally experienced several changes in my commitment to theories and paradigms, which

were prompted either by external motives or self-induced discovery independent of the extant information. These changes occurred either over a period of a year or so, or, more than once, within a matter of an hour — almost like lightning.

So *one day* may be long enough for a scientist to abandon one particular theory in favour of another. In this context and in opposition to Hesketh³, I support the scientists in the British Museum (Natural History) when they write⁴: "the theory of evolution would be abandoned tomorrow if a better theory appeared", although I admit that people would vary in the speed at which they might come to find a novel theory "better" than the present one.

In this connection, I wish to point out that the current trouble with the evolutionary theory does not reside in science itself but in the attitude of some religiously committed people. If so, science will be able to remedy the situation only when it appears trustworthy in the eye of the public. Science is *not* a religion. In contrast to followers of a religion, scientists should admit that the currently held (scientific) theory may very well be wrong⁵, whatever consequences that may have for a civilization based on science and technology.

A. SIBATANI

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Protecting the work

SIR — Many scientists need to handle biological material in a containment facility that provides sterile conditions for the work as well as protection to the operator. This requirement highlights one aspect of the current debate concerning performance and use of microbiological safety cabinets where an increasingly held belief (based mainly on misconceptions as to the properties of particles in moving airstreams) suggests that Class I cabinets can provide considerable product protection in addition to the operator protection for which they were designed, and that consequently there is no need for the sophistication of Class II types.

We have recently carried out a series of experiments, in ordinary laboratory conditions, which clearly demonstrate that protection is not obtained in Class I cabinets whereas in Class II types, built to the requirements of BS 5726 and working correctly, there is a consistently high degree of product protection that is independent of the particulate contamination in the laboratory air.

Although the idea of using a Class I cabinet for some degree of product protection is attractive, it is evident that this is intrinsically unsound and should be abandoned.

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NEWS AND VIEWS

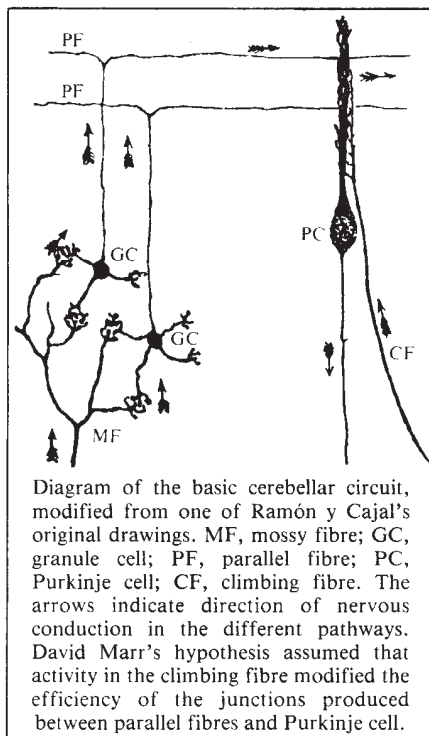
Cerebellar modelling

from Rodolfo R. Llinás

DAVID MARR's untimely death last year is a great loss to neuroscience; he introduced changes in this field that will remain with us for many years to come. Of the areas of work at which he excelled, two of the most prominent are his model of cerebellar function and that of the visual system. His approach tackled some of the truly central issues in neurobiology today. In particular, I refer to the problem of correlating the properties of single neurones and of neuronal nets to the global functions of the brain.

For people interested in establishing such a bridge, the cerebellum continues to be both a thorn in the side and a reason for hope. The basic dogma of the past, that from the diligent study of the electrical properties of single neurones and their resulting nerve nets would arise *ipso facto* a general understanding of brain function, without such unsavoury steps as general hypotheses, is now relegated to oblivion. This turnabout has resulted to some extent from research on the cerebellum where knowledge of its phylogeny, ontogeny, morphology, physiology and neuropharmacology, although most complete, has not *per se* provided new understanding regarding its overall function — this is the thorn in the side. Indeed, although it is fundamentally true that an understanding of brain function must be based on knowledge of the neuronal elements which constitute the brain, as the properties of the system ultimately reside in the properties of the neurones and the nets which they weave, it is also true that such knowledge must be embedded in concepts capable of dealing with the properties of the system as a whole.

One of Marr's basic contributions to neurobiology was the formulation of a global hypothesis regarding the workings of the cerebellum¹. This formulation, which was published in 1969, may be considered one of the first general hypotheses concerning central nervous system (CNS) function to be based on the detailed electrophysiological properties of single neurones. Although other theories regarding the function of this system could be considered early models, such as that of Braitenberg and Atwood², they were basically derived from purely morpho-



logical parameters and, while ingenious and beautiful, were later refuted by more detailed knowledge of electrophysiology brought to light in the mid 1960s. Marr's model, in addition to proposing a broad solution, presented an interesting blend of important ideas.

Fundamentally, the model considered the cerebellum as representing, by the activity of one of its inputs, the mossy fibres, a continuous description of the functional state of the musculoskeletal system and of other brain regions directly involved with the central programming of movement (such as the vestibular system and motor cortex). Marr's view regarding this input was quite close to the classical view in which mossy fibres from many regions of the CNS are considered the main information-gathering system which allows the cerebellar cortex to correct for possible mistakes in motor execution. The

pivotal point here is that motor coordination arises at cerebellar nuclear levels by the combination of two types of nerve terminal. The first type is the collaterals of mossy fibres which wind their way to the cerebellar cortex and end directly on the neurones of the cerebellar nuclei as excitatory terminals. The second consists of the inhibitory synapses provided by the Purkinje cell axons, the only output of the cerebellar cortex. This latter input brings to the cerebellar nuclei an elaboration by the cerebellar cortex of the mossy fibre afferent information. Cerebellar coordination is then considered to arise from the 'comparison' of these two inputs.

With regard to the second cerebellar afferent system, Marr's model formally introduced the attractive idea, originally suggested by Brindley³, that the function of the climbing fibres (which are the inferior olive's input to the Purkinje cells) is to modify the synaptic properties of the parallel fibre–Purkinje cell junctions. The postulate (that of heterosynaptic modification via a postsynaptic element) added to the concept of cerebellar control the very interesting possibility of plasticity on the basis of totally unambiguous morphology. This hypothesis is appealing because it provides a possible clue for the rather monstrous morphological differences between the climbing fibre and the mossy fibre/parallel fibre inputs to Purkinje cells. Indeed, the one-to-one relationship between a climbing fibre and its Purkinje cell (see the figure) is such that several hundred synaptic junctions are formed between the two elements establishing the most powerful chemical synapse in the CNS. This morphological arrangement contrasts vigorously with the mossy fibre/granule cell/parallel fibre system which is the richest input to any cell in the CNS with as many as 250,000 synaptic contacts, each from a different granule cell. These two inputs to Purkinje cells presented a possible example in which plastic synaptic interactions could in fact be expected. Thus, if the activation of climbing fibres could somehow modify the properties of the parallel fibre–Purkinje cell synapse in the sense of increasing the efficacy of the parallel fibre–Purkinje cell

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junction (if the activity of the two inputs were to occur at short time intervals), this model would have gone far in solving one of the central problems in CNS function: that of the acquisition and retention of 'memory engrams'.

Unfortunately, more than ten years after its formal introduction and after much research in this area, Marr's model has fallen from favour on at least two grounds. First and most important, synaptic modification as postulated by Marr — an increased efficacy of the parallel fibre–Purkinje cell synapse by the co-activation with the climbing fibre input — has not been demonstrated. The fervour and tenacity with which this idea has continued to be tested, even without the accumulation of direct experimental evidence in its favour, speak of its seductiveness and beauty. On the other hand, the plethora of claims on indirect grounds for and against Marr's hypothesis (despite the clarity of the postulate and the unambiguous requirement for its experimental validation) is in itself quite telling, and the present decreasing number of positive claims suggests that if modifiability indeed exists, it will relate more to a decrease of the efficacy between parallel fibre and Purkinje cell after climbing fibre activation^{4,5}, rather than the increase in efficacy postulated originally by Marr. Although this is a major departure from Marr's hypothesis, the above claims, if correct, may be considered in accordance with the original proposal by Brindley³. However, direct demonstration of synaptic modifiability has yet to be shown.

Second, at another level, experimental results continue to plague the general hypothesis of cerebellar plasticity. Several lines of investigation have suggested that the cerebellum may be necessary for the acquisition and retention of organized motor responses. One such motor response is the posture and movement compensation which follows unilateral vestibular nerve lesion. This is an ideal paradigm as it involves acquisition of quite complex motor skills and vestibular nerve lesion is accompanied by a total loss of equilibrium. Moreover, because this compensation is required in real life after vestibular damage produced by infectious lesion of the middle and inner ear, it has the advantage of not being experimentally contrived. It was deemed a potential test of cerebellar plasticity on account of its being deeply impaired by cerebellar ablation in the rat. After unilateral vestibular lesion, all vertebrates demonstrate continuous rotation and rolling which would be incompatible with survival unless quickly corrected. The animals must then learn to stop unilateral rotation. Compensation is impossible if the climbing fibre afferents are removed (the climbing fibre input appeared necessary for the acquisition of vestibular compensation); so the paradigm seemed most appropriate to test Marr's hypothesis⁶. A serious problem arose,

however, in testing for the retention of this compensation several weeks after the surgical section of one vestibular nerve. After complete vestibular compensation, inferior olivary lesion leading to climbing fibre death produced an immediate reversal to the abnormal pre-compensated motor behaviour, implying that the learning had not occurred at the cerebellar cortex. If this were to be the case, the parallel fibre–Purkinje cell synapse, having been modified by climbing fibre activity during the compensation period, should have carried on the learned motor behaviour and vestibular compensation should have been unaltered.

In more recent experiments in cats, a similar phenomenon — loss of a learned motor behaviour after an inferior olivary lesion — was confirmed⁷. Reversible blockage of the olivo-cerebellar system by local anaesthetics rapidly abolished a previously learned compensatory eye movement adaptation to reversing prisms. Because inferior olive blockage abolished this learned adaptation without any delay, and because the learned adaptation reappeared immediately after the cessation of the local anaesthesia, it was concluded that no memory engram was present in the cerebellar cortex; rather, the climbing fibre system seems to be a true afferent system, not a modifier of synaptic efficiency at the Purkinje cell. This suggests that the actual modifiable system is not located in the cerebellum, and so the cerebellum must be considered an important centre for the expression, but not the locus, of the actual motor learning.

There are other general models of cerebellar function, some quite similar to Marr's⁸ and others rather different^{9,10}, of

which the differences, virtues and flaws are still being debated. The cerebellum continues to be one of the most dynamic areas of research in neurobiology; it continues to give us new and important clues regarding the function of single cells and the properties of neuronal networks. Furthermore, as we begin to understand the nature of motor coordination — the ability of the brain to transform desired movements (intended movements) into actual movements (motor executions) — the clearer will become the role of the cerebellum in the gamut of brain transactions, and this is the reason for hope! Because some of the models being discussed present formal alternatives to how the cerebellum coordinates movements and because of the theoretical interest which such understanding has in relation to the increasing interest in artificial intelligence and robotics, it is very likely that the cerebellum will continue to offer one of our best chances for obtaining a global understanding of any region of the nervous system. Whatever the outcomes of these efforts, the fact ultimately remains that individuals like Marr appear rather seldom and in losing him we lose more than a good friend and a respected colleague. □

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Grand bang

from Paul Davies

WE are all familiar with three states of matter — solid, liquid and gas. Each phase can be reached by heating the previous phase. This is not the whole story, however, as physicists have discovered new phases of matter that arise when temperatures are raised still further. At several thousand degrees a gas will ionize into a plasma and at several million degrees the nuclei fragment into individual protons and neutrons, while at still higher, as yet uncertain, temperatures, the identities of even these nuclear particles are lost. The behaviour of matter is then determined by the quarks that compose the neutrons, protons and other subnuclear particles.

At each successive phase transition,

some structure is lost. A melted solid loses its crystalline structure, a boiling liquid gives way to a chaotic *mêlée* of molecules, and with higher temperatures the molecules, and eventually the atoms themselves break up. All distinction between atoms disappears when the nuclei fragment until finally, even names such as 'neutron', 'proton' and 'meson' become meaningless.

Now some physicists are speculating about yet another phase of matter, beyond the quarks, which is achieved at the amazingly high temperature of 10^{30} K. Under these extreme conditions, matter is smashed into its ultimate building blocks, and theory predicts that even the quarks and leptons lose their individual identities. The fundamental forces that control the structure of the microworld merge into a single unified force, and physics is reduced

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Dominance hierarchies among worker ants

from Robert M. May

THE pecking order of barnyard fowls and other birds, and the dominance hierarchies of hamadryas baboons and other primates (including academics), are widely known. One tends to think of such phenomena, however, as being found only among vertebrate species.

In a remarkable recent study, Cole (*Science* **212**; 83, 1981) has demonstrated the existence of linear dominance hierarchies among the workers of the ant species, *Leptothorax allardycei*. These dominance hierarchies, moreover, have all the classic characteristics of their vertebrate analogues: routine displays of dominance, avoidance behaviour and even fighting.

Cole collected colonies of these neotropical myrmicine ants from their habitats among dead sawgrass stems in pine and palmetto scrub (in the Florida Keys) and housed them for observation in glass nests. Encounters between worker ants were then watched and the results tabulated in the conventional matrix form; these interaction matrices comprise totals of 200–400 interactions, over 10–20 hours of observation. In Cole's words, the agonistic encounters begin "with the dominant ant touching the submissive ant with its antennae. There is brief pause before the dominant either rapidly turns or lunges toward the subordinate. It then pummels the gaster, thorax, or head of the subordinate with its mandibles. The encounter may continue, with the dominant ant advancing onto the top of the

subordinate, sometimes climbing clear of the nest floor and standing on the subordinate. The typical response of the subordinate is to crouch and freeze, with the antennae drawn back to the sides of the head. It stays in this position until the dominant moves away." The dominance hierarchies thus established are almost perfectly linear, with less than 0.5 per cent of the interactions being 'reversals'. The highest ranking worker accounted for about 43 per cent of all dominance displays, and the second ranking for about 28 per cent; this is typical also of vertebrate dominance hierarchies (see, for example, Wilson *Sociobiology*, Harvard University Press, 1975), and is plausibly explained on the grounds that the higher the rank, the more worthwhile it is to expend effort in defending it.

Cole demonstrates two main advantages that accrue to the dominant workers. First, they are favoured in the exchange of liquid food. Although it is common for liquid food to be exchanged among workers in colonies of social insects, in *L. allardycei* this food transfer is always in one direction. High-ranking workers receive food from low-ranking ones, but do not reciprocate; Cole estimates the probability that chance alone could account for such apparently unidirectional transfer of food to be less than 0.001.

Second, Cole showed that workers laid roughly 20 per cent of the colony's eggs.

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He did this by removing the queen for 3 days, and feeding the workers food in which Sudan black was mixed; Sudan black is fat soluble and not transferred in liquid food exchange, so that the queen is not contaminated with the stain when she is returned to the colony. As a result, the queen's eggs were the normal white colour, while those produced by workers (amounting, on average, to 21.8 per cent of the total) were stained blue. The haplo-diploid sexual system of these social insects means that the workers' eggs (which are unfertilized) produce males; the fertilized queen can produce males or females. Cole further demonstrated that the dominant workers had substantially greater ovarian development, and thus presumably accounted for most of the worker-produced males. In short, *L. allardycei* has effectively two reproductive castes: the queen, who produces all the females, and the high-ranking workers, who produce at least a significant fraction of the males.

The dominance hierarchies in *L. allardycei* constitute a most engaging curiosity. They also hold larger evolutionary implications. As Cole emphasizes: "this social organization . . . has characteristics intermediate between those of the supposed nonsocial ancestors or ants and those of advanced eusocial species. Consideration of the social organization of this species must alter the conception of the reproductive options available to the worker caste of social insects."

to its most basic simplicity. Or so the theorists claim.

Temperatures of 10^{30} K are, to say the least, inaccessible to present technology. There was one occasion, however, when matter could have been as hot as this: during the big bang. The creation of the Universe generated enormous quantities of heat, and it is generally supposed that the temperature of the primeval furnace plummeted from unlimited values at the outset to a mere 10^{10} K within one second. For about 10^{-35} seconds (which is brief even by physicists' standards), the temperature could have been high enough for the predicted ultra-simple phase of matter to have fleetingly existed.

Matter, and the forces that control it, are pictured as having frozen out of the featureless primeval soup in a succession of phase transactions, each with progressively more structure. The distinctive differences between leptons and quarks, and between electromagnetic, weak and strong nuclear forces, are regarded as a purely low-temperature differentiation.

When water freezes to ice, it releases a great deal of heat — so-called latent heat —

which is why water can seem reluctant to solidify at 0°C . Similarly, in any phase transition, heat is either absorbed or expelled to help drive the transition itself, even without any change of temperature. Calculations suggest that when the quark-lepton phase froze out of the primeval furnace, a large quantity of heat was also released.

This most exotic phase of matter has been studied by A.D. Linde of the Lebedev Physical Institute in Moscow. In a recent paper (*Phys. Lett.* **99B**; 391, 1981), Linde argues that the quark-lepton freeze-out was an event of shattering proportions. What elevates the primeval latent heat release to the status of a 'bang' is the phenomenon of supercooling. It is well known that a very pure vapour can be carefully cooled to below its condensation temperature without liquid drops appearing. A slight disturbance will then lead to a catastrophic appearance of droplets. This principle was used in the so-called cloud chambers to detect the passage of subatomic particles.

Similarly in the primeval Universe, the quark-lepton phase transition did not

occur as soon as the temperature fell to the 'condensation' temperature. Because it depends on certain quantum 'tunnelling' processes that require a finite duration, the transition was inhibited until about 10^{-35} s, by which time the temperature was down to a modest 10^{28} K. When the transition finally occurred, in the words of Linde, it took on the features of a great explosion "shaking the whole Universe and changing abruptly its temperature".

The catastrophic release of energy suddenly reheated the cooling Universe and drastically altered the details of its evolution at a most sensitive epoch. Current theories suggest that it was at this stage that the Universe acquired its pre-dominance of matter over antimatter, and that various enigmatic processes occurred which suppressed the production of magnetic monopoles. Linde calls this phase transition 'the grand bang' in honour of the so-called grand unified theories of fundamental forces that predict such a transition. No doubt its reverberations will disturb some of the tidy theories currently being formulated concerning the very early Universe.

Malaria vaccine protects offspring

from F.E.G. Cox

THERE can be no doubt that an antimalarial vaccine is one of the major requirements of tropical medicine and a number of laboratories are attempting to develop one. There is equally no doubt that vaccination against malaria is possible and has been achieved using a wide range of experimental model systems, but the protection produced varies in efficacy and all the methods so far described have serious drawbacks.

In the life cycle of the malaria parasite in the human host, the infective stages injected by the mosquito are the sporozoites, which circulate in the body for about half an hour before entering the liver where a massive phase of multiplication occurs. This results in the production of thousands of merozoites which invade red blood cells where another multiplication phase, erythrocytic schizogony, takes place. The products of this phase are further merozoites which invade fresh red blood cells in which erythrocytic schizogony again occurs and this process is repeated until the host dies or recovers. Eventually sexual stages are produced and these are taken up by and infect the mosquito vector¹.

The various stages in the life cycle differ antigenically from one another; thus as the immunity produced is stage specific, a blockbuster approach to immunization is not feasible. At present the main targets for vaccines are the sporozoites, the merozoites, the erythrocytic stages and the gametocytes². The use of irradiated sporozoites as a vaccine has been largely confined to rodent models but with considerable success. Adult mice can be protected by the intravenous injection of irradiated sporozoites³. The protective antigen is a surface antigen that can be demonstrated with the use of monoclonal antibodies⁴ and monoclonal antibodies against this antigen protect mice against challenge with the homologous species⁵. On page 331 of this issue of *Nature* Orjih and colleagues report that these findings have been extended to young mice and the offspring of immunized mothers.

Briefly, adult mice given one to four immunizing doses of irradiated sporozoites intramuscularly were poorly protected against challenge with live sporozoites several weeks later. Young mice 2–14 days old, on the other hand, were well protected. The fact that adult mice can be protected by the intravenous route but not the intramuscular one is interesting but what these experiments do show is that young animals can be immunized in an acceptable way. Of even greater impor-

tance is the fact that when adult female mice were immunized intravenously with irradiated sporozoites, over half the offspring were protected against challenge and similar levels of protection were found in mice born to normal mothers but fostered onto immunized ones.

These results are particularly important because, in many parts of the world, malaria is essentially a disease of children. In endemic areas, babies acquire immunity passively from their mothers and are then protected during the critical first few months of life. Control schemes, however, upset this balance and it is in such situations that a vaccine that protects young children is likely to be of most use. Another important aspect of these results is that similar findings may apply to other vaccines such as the merozoite one being developed at Guy's Hospital in London², and recently described improvements in the *in vitro* cultivation of human malaria parasites^{6,7} and adjuvants⁸ have moved such a vaccine a further step forwards. The merozoite vaccine is known to protect

monkeys but for obvious reasons the possibility of transfer of immunity to their offspring has not yet been investigated.

Those who have advanced the cause of a malaria vaccine have never suggested that a totally effective vaccine would ever be possible but have argued that a vaccine could produce better protection than a natural infection and could ameliorate the course of the disease. These experiments with *Plasmodium berghei* appear to vindicate this cautious approach. However, before too much euphoria sets in it must be remembered that the passage of immunity from mothers to offspring differs markedly from species to species and what happens in a mouse or monkey need not necessarily happen in man. □

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Continental swells

from Peter J. Smith

As newly formed lithosphere spreads away from oceanic ridges it cools and contracts, leading to a subsidence of the ocean floor that increases generally with age. Indeed, as Sclater and colleagues have shown (*J. geophys. Res.* **76**; 7888, 1971), there is a simple relationship between lithospheric age and water depth, which may be regarded as the first-order description of ocean floor elevation. However, as with most geological phenomena, there are also second-order and even third-order effects — local deviations from the general rule. Chief among these are the broad topographical swells, typically 1,000 km or so across and about 1 km high, associated with zones of extensive intraplate volcanism such as the Canaries, the Cape Verde Islands and Hawaii, as well as the topographical highs representing aseismic ridges such as the Rio Grande Rise and volcanically long extinct regions such as Bermuda.

Cochran and Talwani (*Geophys. J.* **50**; 495, 1977) have recently shown that both

the volcanically active topographical features (known in certain quarters as hot spots) and their inactive counterparts are associated with positive gravity anomalies of intermediate wavelength — an observation that poses certain problems of interpretation, especially if volcanic activity alone is to be regarded as the lithospheric manifestation of mantle plumes. Similar gravity effects suggest common underlying causes; and yet it is not entirely clear how topographical anomalies associated with volcanic activity, whether plume-related or not, could be supported by the same mechanism as, say, aseismic ridges. Detrick and Crough (*J. geophys. Res.* **83**; 1236, 1978) may or may not be right, therefore, in attributing the 'active' topographical swells to reheating of the lithosphere.

Whatever the mechanism may be, what produces little more than a topographical swell in oceanic lithosphere may produce rather more complex results in continental lithosphere. This supposition has led Crough (*Geology* **9**; 1, 1981) to examine the consequences of a possible topographical swell moving across parts of the United

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States and Canada. From the known drift of North America and information about dated igneous intrusions, it is clear that from at least 160 to 100 Myr ago the continent passed northwestwards over the hot spot now near Great Meteor Seamount in the eastern Atlantic. If that hot spot, by whatever means, produced a topographical swell throughout its journey, *a priori* it should have had an influence on continental evolution in terms of elevation, thermal history and patterns of erosion along a track from at least 50°N latitude in Canada southeastwards down to the New England coast.

Crough's case for suggesting that such an influence was indeed exerted begins with the observation that the hot spot trace runs approximately along the northern limit of the Palaeozoic sedimentary cover in eastern North America. To the south of the track lies the Appalachian basin; to the north lies the largely exposed Canadian Shield. There are numerous lines of evidence, however, to suggest that the sediments of the Appalachian basin once extended much further to the north through southern Canada.

First, there is the circumstantial evidence provided by the nature of the existing basin sediments of late Ordovician and younger age, foreland clastic deposits derived from Palaeozoic mountains to the east. It is reasonable, claims Crough, to suppose that these mountains would also have given rise to enough sediment to bury the shield to the west. Second, the present outliers of Palaeozoic (Ordovician–Devonian) sediment in the shield area imply a once more extensive cover. Third, isopach maps of the remaining sediment in the Appalachian basin indicate no thinning towards the north, as might have been expected had the present northern edge of the basin always been the limit of sedimentary cover.

Fourth, lithofacies relations show the present northern margin of the basin to be defined by post-depositional erosion. There is no evidence of a clastic source along that margin, for the clastic ratio of the basin sediments increases from west to east with little north–south variation; and in any case, palaeocurrent indicators generally demonstrate sediment transport towards the north-west and west rather than from the north. Finally, conodont geothermometry establishes that those few sediments remaining in southern Canada and northern New York were once deeply buried, perhaps beneath up to 4 km of overburden.

So what happened to this once more extensive sediment cover to the north? Obviously it was eroded away; and it is Crough's contention that the erosion occurred as the hot spot-induced uplift, at a maximum (and hence producing maximum erosion) along the axis of the hot spot track, moved down from Canada towards New England. In other words, a hot spot moving relative to an overlying

continent can have a far more profound effect than simply generating volcanic activity at the surface — although there is that too, as the kimberlites and alkalic complexes, not to mention the offshore New England seamounts, testify.

The chief information for the timing of the passing uplift and erosion comes from apatite fission-track ages of the basement along the supposed hot spot track. Apatite acts as a maximum thermometer, recording the time at which the mineral last cooled below 100°C, which, if Crough is right, would be the time at which the removal of the Palaeozoic cover allowed cooling from burial temperatures. The many apatite fission-track ages already available from the region are almost all

Cretaceous to early Tertiary and thus consistent with the passage of the Great Meteor hot spot.

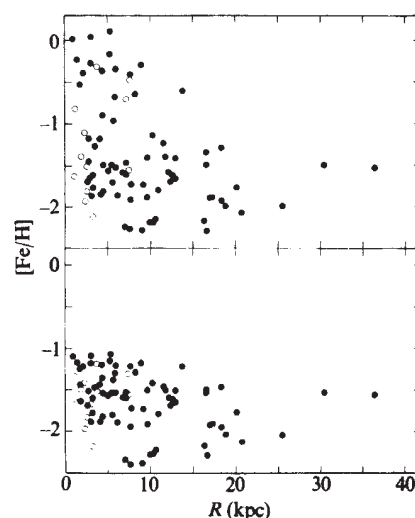
Crough's interpretation differs from some others; Dewey and Kidd (*Geology* 2; 543, 1974), for example, attributed variations along the Appalachians to processes occurring at the time of orogeny. Insofar as Crough's study applies to a particular region, it must be judged by others familiar with the regional geology. But if hot spot-induced continental uplift proves to be common (and only examination of the loci of other subcontinental hot spots will decide this issue), it will presumably force a general re-evaluation of the style, and perhaps timing, of much continental erosion. □

A revision of the globular cluster metallicity scale

from Barry F. Madore

FEW probes of the spatial, chemical and dynamical evolution of our Galaxy have held such a pre-eminent position as have the globular clusters. Being the surviving subunits of the early collapse of our Galaxy, these bright, self-contained systems of old stars have long been used by astronomers to calibrate the size and composition structure of our own and other galactic systems. However, this legendary structure is coming under rapid reappraisal with mounting evidence that the basic calibrations of the metal content of globular cluster stars may be in error.

The collapse of the calibration is a direct result of advances in instrumentation and the observational efforts of Catherine Pilachowski, George Wallerstein, Ron Canterna and others from the University of Washington, as well as from the studies of Judith Cohen at the California Institute of Technology. Hints that the metallicity of the most metal-rich globular clusters in our Galaxy had been overestimated first surfaced at the Advanced Study Institute on Globular Clusters*, and these results were later presented in a refined form (C. Pilachowski, C. Sneden & R. Canterna, p.467; J. Cohen, p.385, *Star Clusters*, ed. J. Hesser, Reidel, Holland, 1980). The most metal-rich globular clusters are now cited as being some five times more metal poor than earlier calibrations had suggested. The calibration for the metal-poor globular clusters has remained relatively unaltered.



The logarithmic metallicities of globular clusters as a function of galactocentric distance, R . The upper panel shows the metallicity gradient obtained when using the old metallicity calibration while the lower panel shows the flattening of this distribution when the newest calibration is applied. Open circles are for globular clusters with uncertain distances. (Taken from *Astrophys. J.* 241; 606, 1980.)

Robert Zinn has reviewed the implications of this revision for models of early galactic chemical enrichment (*Astrophys. J.* 241; 602, 1980). The oft-cited 'metallicity gradient' for globular

*The Advanced Study Institute on Globular Clusters was sponsored by NATO at Cambridge, England in August 1978.

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clusters as a function of radial distance from the centre of our Galaxy is shown in the upper panel of the figure; this is using the old metallicity scale. The gradient is clearer in the upper envelope of this distribution. At face value this plot indicates that the most metal-rich globular clusters preferentially inhabit — and by implication, were preferentially formed in — the inner regions of our Galaxy. Metal-poor systems formed in a more extended (earlier) configuration. These data supported the canonical collapse theory of a well mixed galactic enrichment model. However, as illustrated in the lower panel of the figure, the situation is significantly modified by adopting the new metallicity scale: the gradient is practically eliminated and with it the simple theories of galactic collapse.

Even without the gradient, one is still left with a significant spread in metallicity at any radial distance and even among globular clusters of the same metallicity

there is the well known problem of 'the second parameter phenomenon'. This phenomenon is manifest by the fact that although some aspects of the distribution of globular cluster stars in the luminosity-temperature plane (such as the height of the cool red giant branch) are a strong function of metallicity, other features (such as the relative population of stars on the red and blue side of the horizontal branch) are independently a function of some as yet unspecified parameter.

Zinn has attempted to provide a unifying solution to these problems using a modified version of a model of galaxy evolution recently proposed by Leonard Searle and himself (*Astrophys. J.* **225**; 357, 1978). In a model of cluster formation and destruction, which encompasses the build-up of the galactic disk, the formation of satellite galaxies to our own Milky Way and the deposition of a retinue of surviving globular clusters, Zinn suggests that the second parameter might be age. If so, the

clusters in the outer parts of our Galaxy would be some three billion years younger than those in the central regions — a situation almost completely opposite to earlier thoughts on the matter.

The issue is certainly not closed and more models, both of individual globular cluster evolution and the formation of the galactic system, are certain to be forthcoming. Indeed, a recent theoretical paper by Demarque and McClure (*Astrophys. J. Lett.* **242**; L5, 1980) concludes, "through a process of elimination", that helium abundance, not age, is a dominant second variable in globular cluster evolution. While high helium abundance would replace older ages for the globular clusters, with this interpretation would come the need for a source of helium abundance variations. A generation of supermassive stars could provide this extra helium at various places in the galaxy but the ultimate reasons for the systematics of their occurrence have yet to be speculated on. □

Cortical effects of monocular deprivation: suppression or deafferentation?

from J. Anthony Movshon

It is eighteen years since Wiesel and Hubel¹ showed that depriving a kitten of vision in one eye causes that eye to lose virtually all effectiveness in activating visual cortical neurones. The developmental mechanism underlying this shift in cortical eye dominance has long been known to depend on a competitive interaction between the eyes², but the neurophysiological basis of the deprived eye's inability to influence cortical neurones remains a matter for debate. On the one hand, there is abundant evidence that monocular deprivation causes a radical reduction in the extent of cortical terminations of afferents originating in the deprived eye³. On the other hand, there is reason to suppose that residual inputs from the deprived eye remain, but are functionally suppressed by inhibitory influences from the normal eye⁴.

A mechanism that could be responsible for this suppression is intracortical inhibition mediated by synapses using γ -aminobutyric acid (GABA) as a neurotransmitter⁵. A few years ago, Duffy and his colleagues⁶ claimed that intravenously administered bicuculline (a GABA antagonist) could reveal an important input from the deprived eye in monocularly deprived cats. They proposed that a GABA-mediated inhibition contributed significantly to the cortical effects of monocular deprivation. There were, however, a number of important defects in these experiments — most

obviously, intravenously administered bicuculline may act at many sites other than the cortical one in question. It is thus welcome that Sillito and his colleagues (*Nature*, this issue, p.318) have now repeated Duffy's experiment using the much more sensitive method of microiontophoresis to deliver minute quantities of bicuculline to the immediate vicinity of the recording electrode. Sillito and co-workers also control for the possibility that it is not the block of GABA *per se*, but rather the overall increase in excitability that results from it, that might unmask the deprived eye's input. Their results show that in a substantial minority of neurones recorded from monocularly deprived cats, bicuculline reveals a GABA-suppressed input from the deprived eye; this input rarely matches and never exceeds in effectiveness the input from the normal eye.

Although Sillito's results seem to support a suppression model of the cortical effects of deprivation, a separate study from his laboratory⁷ makes it unlikely that this suppression is specific to deprived cats: apparently even in normal cortex, about half the monocularly activated neurones change eye dominance during bicuculline administration. It would seem that whatever mechanisms mediate this inhibition in normal cats continue to function in deprived cats, and that the inhibition is not a consequence of the deprivation.

There remain some important issues addressed neither by Duffy's group nor by Sillito's. The most important of these concerns the laminar location within the

cortex of neurones that show suppression effects. The anatomical data concerning the redistribution of afferents from the two eyes after deprivation apply only to layer IV, the layer within which the bulk of the specific afferents to cortex terminate. In normal animals, the eye dominance of a 'column' of cortical tissue — extending both above and below layer IV — is determined largely by the eye dominance within the adjacent patch of layer IV. In deprived animals, however, eye dominance outside layer IV may depend on other factors; deprivation of late onset can cause marked changes in cortical dominance outside layer IV without altering the distribution of inputs to layer IV⁸. It thus appears that two mechanisms of deprivation must exist, one that determines the distribution of afferents within layer IV, and a second that determines the relative effectiveness of the output from different patches of layer IV to other cortical layers. The extra-layer IV mechanism might be, in part, a suppressive one, and it is thus important to know the laminar distribution of neurones showing GABA-mediated inhibition from the normal eye. Moreover, a difficulty arises if this suppressive interaction is confined to particular layers: interocular suppression effected in one layer might have a

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powerful influence on neurones — recorded in other layers — that receive input from the 'suppressed' layer. Yet microiontophoretically applied bicuculline would not spread far enough to be effective in layers remote from the recording site. Thus Sillito and colleagues' estimate of the proportion of neurones influenced (directly or indirectly) by GABA-mediated inhibition might well prove too low.

In any case, it is evident that both the redistribution of afferents within layer IV and a degree of interocular suppression

(perhaps outside layer IV) contribute to the cortical effects of monocular deprivation. While the available evidence suggests that interocular suppression plays a secondary role, resolution of the issue must await detailed study of the laminar location and intracortical connectivity of 'suppressed' neurones, as well as attention to potential inhibitory neurotransmitters other than GABA. Plainly, Wiesel and Hubel's straightforward observations of eighteen years ago opened the door on complex features of cortical organization.

1881), equalling the value for 1944. The next three warmest winters are, in order, 1926, 1958 and 1980. In the past two years we have, therefore, experienced two remarkably warm winters, contrasting sharply with the winters of 1969 and 1972 which were the two coldest winters since 1917. For the 1981 winter, especially in January and February, the maps published by the Free University of Berlin (*Berliner Wetterkarte*) show remarkable warm anomalies over northern Canada and Alaska, and over north-central Siberia — with a much smaller-magnitude cool anomaly over the North Atlantic. Many places in northern Canada and Alaska recorded record warmth in January, some places being more than 16°C above the 1931–60 'normal'.

The warmth of the past two winters may be reflected in Northern Hemisphere snow cover. In a recent paper in *Nature*, Matson and Weisnet¹⁰ described changes in snow cover, based on satellite measurements, over the period 1967–1980. Over North America, a rising trend up to $16.9 \times 10^6 \text{ km}^2$ in 1979 was followed by a sharp drop in 1980 ($15.1 \times 10^6 \text{ km}^2$). The 1981 value is even lower, $13.9 \times 10^6 \text{ km}^2$, the lowest on record. For Eurasia the 1980 and 1981 values are both low (but not as low as the 1970 value), while for the whole hemisphere the values for 1980 and 1981 are $38.9 \times 10^6 \text{ km}^2$ and $38.0 \times 10^6 \text{ km}^2$, values surpassed only by the 1970 value ($37.2 \times 10^6 \text{ km}^2$) (1981 data from Michael Matson, NOAA/NESS, Washington, DC 20233). In spite of the correspondence between recent winter warmth and low snow cover, the overall correlation between these two variables is low. For the first 13 years of record the correlation coefficient between hemispheric winter snow cover and temperature was -0.03 . When 1980 and 1981 are included this rises to -0.40 !

It is difficult to know the significance of these recent events, but it is certainly premature to associate them with the effects of increasing atmospheric carbon dioxide. At present, the high inter-annual variability of surface temperatures (that is, the 'noise' of natural climatic variability) makes it impossible to identify the apparent trend with any particular causal mechanism. We note also that a similar warming trend has occurred before, between the late 1910s and around 1940. However, a few more consecutive warm winters, with continuing warmth in the other seasons, will doubtless give climatologists pause for thought.

Global warming?

from T.M.L. Wigley, P.D. Jones and P.M. Kelly

In 1975, Lamb and his colleagues¹ noted that the downward trend of Northern Hemisphere surface temperatures, which had begun in the early 1940s, had come to an end — at least in the northern North Atlantic. Since then, the direction of the global temperature change has been a controversial issue. Some argue, justifiably, that we cannot be sure of global-mean conditions because there are such large gaps in the data coverage, particularly over the ocean and in the Southern Hemisphere. However, it can be argued that, because temperatures are more uniform over the oceans than the continents, large oceanic data gaps can be tolerated (see Barnett² for an opposing view). Further controversy lies in whether or not surface temperatures are meaningful at all. Perhaps a better measure of large-scale fluctuations in atmospheric temperature is the column-mean temperature, as determined, for instance, by the 1,000 to 500 mbar height difference. Doubts have been expressed by Parker³ as to the reliability of these data but, provided the limitations are taken into account, considerable insight into global temperature changes can be gained.

Much useful information can be gained from recent Russian publications. Borzenkova and his colleagues⁴ gave a series of annual mean temperature values averaged over the latitude belt 17.5–87.5 °N for the period 1881–1975 which show a marked warming since 1964. Despite criticisms⁵, Vinnikov⁶ has now convincingly shown that the data are homogeneous and do not suffer from discontinuities apparent in earlier work. Vinnikov also pointed out that the warming trend they had detected was certainly not spatially 'universal' ...

'variations of air temperature at the surface of the Earth may have different signs over extensive areas of the globe in specific seasons of the year, even against the background of a significant rise in average global characteristics' (p. 97). Thus, while an overall warming may have occurred, quite large regions may still exhibit a cooling trend. Although the trend revealed by Borzenkova appears, therefore, to be well established, one comment⁵ must be accepted — that we do not know whether the warming is liable to continue into the future. All of this, of course, begs the questions of what is meant by 'trend', and how one determines the beginning of a trend, given the large year-to-year variability.

What has happened since 1975? A recent Russian paper⁷ gives results up to and including 1978, and we⁸ have independently made estimates of Northern Hemisphere average surface temperatures up to February 1981. Our own results, based on very much the same data as used by the Russians, naturally correlate highly with theirs. The data show 1972, 1974 and 1976 to be quite cold years, agreeing with the estimates of Barnett² and Angell and Korshover⁹. In fact, the data show 1972 to have been the coldest year since 1917. Vinnikov *et al.*'s data agree for 1972 and 1976, but they show 1974 as a warm year. A cooler 1974 and a cool year in 1976 reduce the magnitude of Borzenkova *et al.*'s 1964–1975 warming trend. However, 1977 was a warm year (the warmest since 1953) and both 1979 and 1980 were also relatively warm. The warming trend appears, therefore, to be continuing; but conditions are still considerably cooler than the balmy period between the late 1930s and the early 1950s.

Perhaps the most outstanding feature of recent years has been the 1981 Northern Hemisphere winter (December 1980–February 1981). This was the warmest winter on record (records began in

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Why mammalian gametes don't mix

from J. Michael Bedford

THROUGHOUT the Metazoa the reproductive isolation of a species is consistently reflected in the restricted affinities of sperm and egg. The possibilities for interspecific fertilization and the precise mechanisms that restrict this among mammals, including man, have been less than clear, but an understanding of the basis of this restriction is gradually emerging. An ability to dissect or overcome the specific barriers to cross fertilization could help us come nearer to a precise analysis of the mechanisms of the normal event. More practically, it may also soon offer an avenue for clinical evaluation of the fertilizing ability and even the chromosomes of human sperm. Considering the sequential steps of the conception process allows us to summarize what we know of the factors that prevent easy union of sperm and eggs from different mammals, and why further study of this should be rewarding.

The exact way that a specificity of sperm and eggs is achieved may vary. Sperm of teleost fish, for instance, must negotiate the specialized environment created around a small hole in the egg shell. Usually, however, organisms practicing external fertilization seem to depend primarily on elements in the egg coat and sperm surface whose 'fit' dictates a specificity of penetration; one that for sympatric marine species prevents the sea becoming a gamete mixing pot. Since the behavioural preliminaries to ejaculation and insemination could preserve a specificity of fertilization in mammals, an extreme individuality of the character of their gametes might seem unnecessary. Nonetheless, despite changes in the character of both gametes in evolutionary transition from external fertilization to the rather complex interaction that characterizes fertilization in placental mammals, such an individuality persists. Cross-species fertilization can occasionally follow artificial insemination, or the mating that sometimes occurs in confined situations between related mammals. Further development then depends on the harmony of gene expression in the embryo and on its intrauterine relationship with the mother — one favoured possibly by the superficial rather than the intimate type of placenta. Such embryos often die during pregnancy (ferret x mink; goat x sheep; rabbit x hare), but others survive to adulthood as healthy individuals that may be wholly sterile (horse x zebra) or not (ox x bison). In general, however, mammalian gametes tend to express their specific character at several points in the process of conception. As a consequence, fertilization will not occur readily even between members of the same Family such as the rat and mouse, let alone among mammals that differ more widely.

Preliminaries in the female. Incompatibility could first be expressed in the foreign tract by failure of sperm to be transported to the site of fertilization in the Fallopian tube, to survive as viable cells, or to be capacitated (mammalian sperm are not able to fertilize immediately after ejaculation and must undergo a final maturation, capacitation, in the female tract). Little is known of sperm function in the native tract, and so the factors affecting foreign spermatozoa are hardly definable. There is no clear pattern to their longevity: occasionally it can be prolonged beyond that seen in the homologous tract (rabbit spermatozoa in the estrous rat

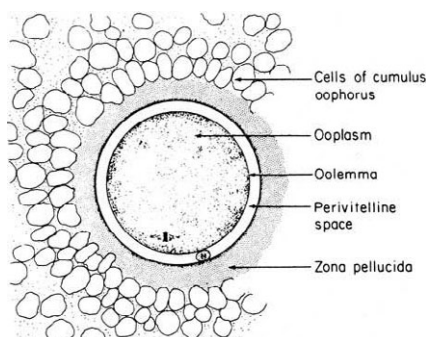


Fig.1 Diagram of a typical mammalian egg soon after ovulation that illustrates the various vestments through which the sperm must pass before being incorporated into the ooplasm.

uterus), but foreign spermatozoa more often die before they would in a native situation and before native spermatozoa in the same uterus or oviduct. Nor does the persistence of motility guarantee transport to the site of fertilization. As shown by the behaviour of human spermatozoa inseminated into the rabbit, maintenance of their motility in the vagina is no ready passport through the cervix to the uterine cavity. The Fallopian tube likewise tends to select the native male gamete from a mixed population of motile cells placed in the uterus, and one can only suspect involvement of the specific surface character of the sperm cell at either point of selection in the tract. On the other hand, capacitation does not appear to be an obstacle when motility is preserved and spermatozoa can experience the tubal environment. Moreover, the time required for capacitation seems more a function of the specific identity of the sperm than of the environment.

Sperm penetration of egg vestments. For gamete union to occur, sperm must first pass through the substantial barriers around the

newly ovulated egg (see Fig.1). The specificity of the immediate preliminaries to the union of mammalian gametes is difficult to elucidate. At the site of fertilization, little is known of the nature of the elements that promote the acrosome reaction, the role and specificity of action of acrosomal enzymes, and the exact function of the specific binding between the reacted sperm head and zona surface. Whether once capacitated, spermatozoa are then triggered to react by a specific signal or do so spontaneously is not resolved. The response of capacitated spermatozoa around foreign and native eggs in the same milieu might illustrate whether the egg coat provides a specific reaction (as it does in sea urchin) and if not, whether the cumulus/corona cell complex expresses a selective resistance towards reacted foreign sperm. That the zona pellucida does so is impressively illustrated by the many successes in interspecific fusion that follow its removal. How it exerts this barrier role still eludes us, again because the precise mechanism of homospecific penetration of the zona is largely unexplained. There is an element of 'Catch-22' in that uncapacitated spermatozoa (which cannot undergo the acrosome reaction) will often attach to the zona pellucida non-specifically in associations incompatible with penetration; but in attaining the capacitated state (in which they undergo the acrosome reaction), sperm probably undergo a surface change which imposes specificity in attachment/binding between the zona substance and sperm plasmalemma. At all events it is probable that specific entities, possibly glycoproteins, are exposed on the surface of the capacitated sperm cell membrane overlying the acrosome, intact or reacted — and that these bind effectively to complementary elements at the surface of the zona pellucida. In the mouse, one of three major glycoproteins that form the zona pellucida is implicated as the receptor through which specific or functional ligands are established. Is the tenacity of this binding the essential key? The answer must await resolution of the means by which spermatozoa normally penetrate the zona substance. The thrashing bound spermatozoon may need the stable union of the reacted peri-acrosomal plasmalemma with the zona surface as a base from which to intrude into the substance of the zona. It does not seem good sense, however, that the now-exposed inner membrane of the acrosome should bond with receptors throughout the zona, since that surface must oscillate and slide freely in cleaving a penetration slit. Neither of the prime factors being considered — a motive force imparted by the

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vigorous tail, and lytic action of residual acrosomal enzyme(s) — carries an obvious element of specificity.

Sperm-egg fusion. Examples have been reported (white-foot deermouse spermatozoa with rat eggs) in which spermatozoa penetrating the zona pellucida then fail to fuse with the mature oolemma. However, the general role of the zona rather than the oolemma as a prime controller of interspecific fertilization is evident from the wider interactions that follow zona removal. Fusion in eutherian mammals differs from that in other animals, the fusion site being a restricted region of sperm plasmalemma over the stable equatorial segment of the acrosome and not the inner acrosomal membrane as in invertebrates and the few other vertebrates studied. However, these special features are maintained in interspecific fusion among mammals and, according to species, removal of the zona simply destroys much of the exclusiveness of the oocyte, allowing combinations as unlikely as pig with hamster. This does not imply open house at the oolemma. Although the case in the hamster, it is not so in other species which show a variability without clear rules. Neither are the interspecific affinities necessarily equivalent for both gametes. Zona-less mouse oocytes display little general plasticity and are penetrated rarely by rat sperm, yet the reciprocal cross is achieved with much success. Fusion with a foreign sperm generally leads to 'activation' of the egg and completion of the second maturation division of meiosis. However, activation is not an invariable sequitur of fusion and it may fail in certain combinations (for example, rat or hamster spermatozoa associating with mouse eggs, or hamster spermatozoa with rat eggs), though why is not known.

Nuclear events. Is there a specificity also to sperm chromatin and the events that bring its transformation in the post-fusion phase? The answer is a qualified no. Unlike histones, the basic nuclear proteins of spermatozoa are not highly conserved, differences of composition being evident even within the Eutheria. Nonetheless, sperm nuclei in foreign oocytes tend to decondense and often complete the process that transforms them to a large pronucleus appropriate for union with that of the egg. Then the male pronucleus may closely resemble that of the host; for instance, when formed from the pig sperm head in the hamster oocyte it visibly mimics the pronucleus formed there by the hamster spermatozoon. This is perhaps not surprising since autoradiographical and immunological studies suggest that pronucleus formation involves a substitution of sperm protamine by proteins contributed by the egg. There are exceptions that imply a specificity of male pronucleus development; for example, rabbit sperm heads show little response once inside mouse eggs. Though untested, inhibiting factors that might operate in

such instances include an absence of egg activation, failure to disperse the investing nuclear membrane and rostral shroud of acrosome and oolemma, or direct incompatibility of sperm chromatin and ooplasmic decondensation factor(s).

Human genetics. Although a viable hybrid has resulted from the mating of a siamang with a gibbon, two closely related apes, there is little known of the interspecific possibilities among primates other than man. The limits of the receptivity of the

will attach avidly, however, to the oocyte of the gibbon, *Hylobates lar*, and when capacitated will penetrate the granulosa vestment and zona pellucida. It might be expected that a similar compatibility exists with and perhaps between other hominoid apes, but the developmental potential of ape oocytes penetrated by human spermatozoa is unknown. Experience from other hybrids involving relationships similarly distant to that of man from any of the apes suggests their early failure might be anticipated.

Clinical uses. Clinicians now seek means of assessing directly the functional quality of the human ejaculate. This would be of great value in diagnosis of the male role in suspected infertility and in evaluating the efficacy of potential male contraceptives that do not deplete the ejaculate of sperm. Although *in vitro* fertilization tests omit a critical element — assessment of the ability of sperm to reach the fertilization site in a viable state — there is great interest in developing this approach to human sperm function. The limited affinities of human sperm mean that the only two options are intact human follicular oocytes that have no capacity for development and/or the zona-less oocyte of the hamster. The first has been shown to be a usable though difficult system in which to test the aspects of human sperm function needed for zona penetration. In the second, an ability of human spermatozoa to undergo an acrosome reaction, fusion and then male pronucleus formation is reflected faithfully in their interaction with the zona-less oocyte of the hamster (though not of mouse or rat).

The human/hamster hybrid system appears a serious possibility for diagnosis of sterility. In some couples with otherwise unexplained infertility, sperm of the male partner fail consistently to enter hamster oocytes after *in vitro* capacitation, even though the suspect sample cannot be distinguished by standard examination from fertile semen. The sensitivity of that system can be increased, moreover, by marking the sperm of the suspect and the normal control samples with different innocuous fluorescent dyes, permitting direct comparison of their fertilizing potential. Unfortunately, while a negative result is of value, the lack of any test of zona penetration makes a positive finding — sperm entry into the zona-less hamster oocyte — an incomplete indicator of sperm normality. The limited evidence supports this last view in that an ability to enter the hamster oocyte and to penetrate the zona of the human follicular oocyte are not always shared; human sperm samples performing in either assay do not always do so in both.

It may prove at least as significant clinically that in human/hamster hybrid fusion the chromatin of the human sperm head can be transformed to a normal male pronucleus and beyond. Culture in the presence of colcemid, an inhibitor of

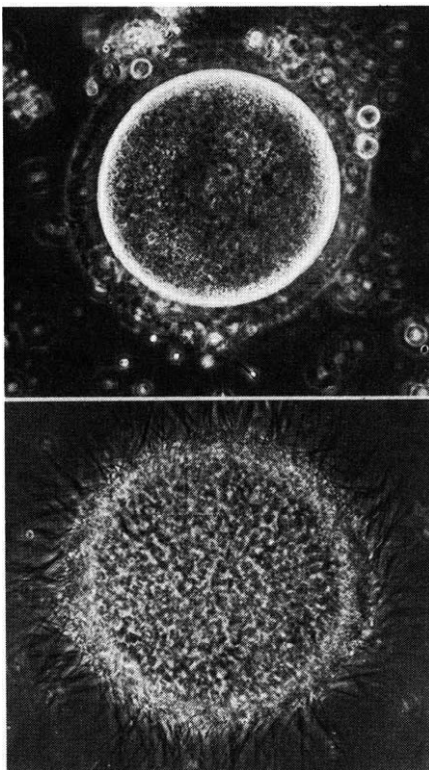


Fig.2 This shows a baboon oocyte (top) and a gibbon oocyte (bottom) that have each been exposed to a similar concentration of human sperm in the same conditions. Capacitated or not, human sperm show no affinity for the zona pellucida of the baboon — only a few sperm are lying adjacent to the zona in a random fashion; by contrast, they adhere avidly to the zona of the gibbon — a true ape — and when capacitated will penetrate the zona.

human oocyte are essentially untested, but while more is known of human spermatozoa the specificity of their function offers little for the fantasists of science fiction or of zooerasty. The evolution of man (there is little evidence for other hominoids) has been accompanied by restrictive change in the affinities of the sperm surface, limiting to an unusual degree the substrates to which it will adhere, capacitated or not. Whereas non-specific attachment of uncapacitated spermatozoa can occur in many mammals, human spermatozoa seem to display almost no affinity for the foreign zona pellucida, even that of sub-hominoid primates such as baboon (see Fig. 2), rhesus and squirrel monkey. Human spermatozoa

spindle formation, has been shown to allow the preparation of the karyotype of the penetrating spermatozoon (variably more than one per egg), of a clarity sufficient for evaluation of the numbers and the character of the sperm chromosomes. With wider establishment of this technique, studies of the possible incidence with which spermatozoa act as primary agents of the chromosome imbalance so

common in early human embryopathies, and the degree to which environmental hazards can affect sperm chromosomes, would seem to be on the horizon. Whatever the conditions of culture, however, the 'hamster' zygote fails after one cell division at the two-cell stage. Even in a world where bacteria are coerced to produce human insulin, small boys with furry brown ears are not a foreseeable prospect! □

laryngeal papilloma may be an example of this¹².

The increase in NK activity mediated by interferon results at least partly from recruitment of previously inactive cells. But interferon also affects target cells: they become more resistant to lysis and have been shown to express more major histocompatibility complex (MHC) antigen. This latter effect, while it may not account for the change in susceptibility to NK cells, might well make virus-infected cells more susceptible to T cell-mediated killing because of the importance of MHC antigens as target structures for T cells.

This raises the question of whether the function of NK cells need necessarily be the killing of tumour cells. Other data suggest a regulatory role: interferon may be produced by NK cells themselves, and play a part in the generation of suppressor T cells¹³. This implies that NK cells are an integral part of the immunological network of T and non-T cells. Since immunologically active cells are regulated by multiple signals (MHC antigens, specific antigens, helper and suppressor factors), it is not surprising that NK regulation has now been shown to depend on signals other than interferon: Henney *et al.* (this issue of *Nature*, p.335) have shown that their activity is augmented by interleukin-2. Whether this is a direct effect or mediated indirectly via another cell type is not established. It thus remains possible that the final mediators of the augmentation are interferons produced by intermediary cells induced by interleukin-2. This would imply synergistic effects of different interferons on NK cells. Irrespective of the details of the mechanism of the interleukin-2 effect however, this finding once again places the NK cell firmly in the immunological network.

In summary, recent data seem to be leading away from earlier and rather sterile arguments about the phenotype and effector specificity of NK cells (although these remain important unresolved questions) and towards a deeper understanding of the biology of NK cells *in vivo* and *in vitro*. The new tools available, monoclonal antisera¹⁴, *in vitro* grown normal¹⁵ and tumour¹⁶ cell lines and purified mediators, should allow rapid progress. □

Understanding natural killer cells

from Peter Beverley

NATURAL KILLER (NK) cells were first identified by their ability to kill, without deliberate prior immunization, certain tumour target cells grown *in vitro*. Otherwise they were defined only in negative terms: that is, they were not thymus-derived (T) or bone marrow-derived (B) lymphocytes, nor were they adherent or phagocytic. Though their origins were thus rather mysterious, they were at first widely presumed to be the mediators of immunosurveillance against tumours¹. Subsequent studies have suggested however that NK cells may be related to either the T² or myeloid³ lineages and may have an immunoregulatory role⁴. Furthermore, several authors have demonstrated heterogeneity of NK effector cells, and that different target cells are differentially susceptible to these effector subpopulations⁵. In the face of this apparent confusion can it now be said that there is such an entity as an NK cell and is it possible to define its biological role?

Examination of mutant animals deficient in various cell types, such as the nude or beige mouse², or of humans with genetically determined diseases⁶, has not settled the controversy over the origin and phenotype of NK cells. On the other hand, NK cells do have specific properties not characteristic of other lymphocytes. In the mouse NK cells have been identified by a serological marker⁷ and in man, although no unique surface marker has yet been defined, the more old fashioned, but not to be despised, use of cytological examination has revealed that most NK cells are large, granular lymphocytes with characteristic cytochemistry⁸. This is not to say that all cytotoxicity detected using cultured tumour lines *in vitro* as targets is due to this cell type. Certainly, activated lymphocytes which carry undoubted T-cell markers will lyse a variety of cultured target cells.

If many different cell types can lyse cultured tumour targets *in vitro* and if freshly isolated cells are in general insensitive to NK activity, what can be made of claims that NK cells operate *in vivo* as a surveillance mechanism against tumours? Several authors have shown that NK cells can mediate resistance *in vivo* against transplantable tumours. Many of these experiments are, however, difficult to interpret because NK cells cannot be purified. Kasai *et al.* (this issue of *Nature* p.334) have now adopted a rather different approach: they have used systemically administered antibody to asialo GM1 surface membrane glycolipid to block NK activity and shown a dramatic reduction in resistance to syngeneic tumour. The relevance of their results to natural tumour immunity is not entirely clear because they used a well established transplantable tumour cell line. The difficulty with such cell lines is that they generally contain tumour viruses and it may be against the viruses that the immune response is directed.

Another line of evidence that could be interpreted as indicating a role for NK cells in protection against virus infection is the observation that NK activity is increased by interferons⁹. Furthermore, antibody to interferon has been shown to block NK function in nude mice, and to allow the growth of xenogeneic cell lines even when these were persistently infected with viruses¹⁰. Such data are highly reminiscent of much older findings in mice immunosuppressed for long periods with anti-lymphocyte serum in which the tumours which arose were shown to be due to polyoma virus¹¹. Taken together, these results may be more consistent with a view of the NK cells as a surveillance mechanism predominantly directed at viruses not tumours. This line of reasoning suggests that therapeutic manoeuvres, such as administration of interferon, which may activate NK cells, will be most effective against virus-induced tumours. In man, the successful use of interferon to treat

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ARTICLES

Demise of the Lemaître cosmological models and objects at large redshift

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Recent uniform samples of QSO Ly α absorption lines make possible new cosmological tests out to large redshifts and the detection of superclustering out to redshift $z \sim 3$. One test involves the Lemaître models of the Universe, which predict large peaks in the number of objects per unit redshift, near the redshift of their characteristic quasistatic period. No such peaks are seen in the Ly α cloud distribution.

AMONG the various standard isotropic homogeneous Friedmann–Robertson–Walker cosmological models^{1,2} with non-zero cosmological constant, the Lemaître models are the most distinctive because of their long quasistatic or coasting period during which the Universe scarcely expands at all (Fig. 1). Light passing through a coasting period which lasts a long time will traverse a large distance, and may intercept many absorbers for a corresponding small change of redshift z . Thus in Lemaître models one expects to observe a pronounced peak in the number of objects observed per unit redshift, near z_s , the redshift of the quasistatic period³.

The Lemaître models of interest all have positive space curvature and a negative q_0 . They are characterized by a positive cosmological constant Λ (repulsive force²), which is fractionally larger than the value Λ_c for an Einstein–static universe given by the relation:

$$\frac{\Lambda}{\Lambda_c} = 1 + \varepsilon = \frac{27\sigma_0^2(\sigma_0 - q_0)}{(3\sigma_0 - q_0 - 1)^3} \quad (1)$$

where the dimensionless density parameter $\sigma_0 = \Omega_0/2$, and ε is a free parameter in the range $0 < \varepsilon \ll 1$. The expected number of absorbing objects per unit redshift along a line of sight is given by⁴:

$$N(z) = cH_0^{-1}\phi(z)\Sigma(z)(1+z)^2|\text{RAD}|^{-1/2},$$

clouds observed per unit z

$$\text{RAD} = (H(z)/H_0)^2 = 2\sigma_0(1+z)^3 + (1+q_0-3\sigma_0) \times (1+z)^2 + \sigma_0 - q_0 \quad (2)$$

where H_0 is the local ($z=0$) value for the Hubble 'constant' $H(z)$, $\phi(z)$ is the space density of absorbing clouds per unit co-moving volume and the mean cloud cross-section at some critical column density is given by $\Sigma(z)$. If the clouds do not evolve then $N(z)$ depends only on the cosmological redshift–distance relation giving:

$$N_{\text{cos}}(z) = N_0(1+z)^2|\text{RAD}|^{-1/2} \quad (3)$$

where N_0 is the local value of $N(z=0) = cH_0^{-1}\phi_0\Sigma_0$. The redshift z_p of a peak in $N(z)$ is determined by the values of σ_0 , $\varepsilon(q_0)$ in equations (1) and (2). A large density σ_0 is required to give low z_p .

$N(z)$ peak test using galaxies and QSOs

Complete and unbiased samples of the redshift distribution of objects are required to search for $N(z)$ peaks. Several peaks have in fact been reported^{5,6}, but these are now considered to be purely the result of observational selection effects.

For $\sigma_0 > 1.14$ and small ε , peaks lie at $z_p < 0.5$ where galaxies are directly observable. Current surveys are only complete to bright magnitude limits (for example, $m_I \leq 15$ in ref. 7), and

contain few galaxies with $z > 0.1$. In addition the redshift distribution is dominated by cosmologically small-scale (≤ 20 Mpc) galaxy clustering⁷, making samples unsuitable for searches for Lemaître peaks.

To extend the test to Lemaître models with lower densities we must use surveys of objects at large redshift. For $\sigma_0 < 0.25$, $z_p \geq 1$ and for $\sigma_0 < 0.05$, $z_p \geq 2$, while at the lower limit for the density of the Universe based on observed galaxies Gunn and Tinsley⁸ choose $\sigma_0 \geq 0.01$ giving $z_p \leq 3.8$. Only QSOs, other radio sources and probably X-ray sources are directly observable at these redshifts.

Large numbers of QSOs are now being discovered by low-dispersion spectroscopic surveys⁹. These surveys are strongly biased towards the detection of QSOs at emission redshifts for which at least two strong emission lines lie in the observational window and consequently show a strong peak in the number of QSOs at $z \sim 2$. This peak, and a narrow component in it at $z = 1.95$, have previously been regarded⁶ as evidence for Lemaître models with $\sigma_0 \sim 0.05$. However, these peaks are adequately explained as selection effects^{9,10}, as is testified by their absence from samples of QSOs selected from radio surveys¹¹.

Aside from the above selection effects, all surveys of the distribution of objects in redshift which are based on the apparent observed magnitude of objects, in any waveband, will be strongly biased towards detecting fewer objects as redshift increases due to the apparent (not absolute) faintness of distant objects. Current samples of objects based on the flux emitted by each object are then inadequate for tests of the presence of Lemaître model $N(z)$ peaks³. It is in comparison with these data that QSO absorption lines provide a very powerful new data base for cosmological tests.

QSO absorption lines

Two major classes of intervening narrow line absorption clouds have now been distinguished in QSO spectra⁴: the metal line

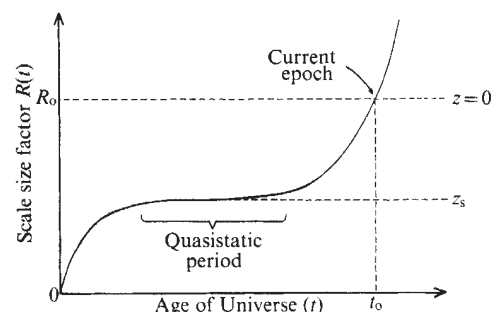


Fig. 1 Scale size of the Universe as a function of time since the Big Bang for a Lemaître model.

systems, and the far more numerous Ly α clouds. It seems probable that a large fraction of the metal line systems arise in the outer halo regions of galaxies which happen to lie along the line of sight¹²⁻¹⁴.

Working with an extremely heterogeneous sample of 136 metal line systems from 637 QSOs, Canuto and Owen⁵ have found a broad peak in $N(z)$ near $z = 2$ which they interpret as evidence for a Lemaître model with $\sigma_0 = 0.05$, $\epsilon \sim 10^{-3}$. In fact this peak probably also has the same origin as that in the distribution of the spectrally discovered QSOs themselves.

To minimize the extent of selection effects in samples of absorption line systems, Young *et al.*¹³ propose strict criteria which each individual system must satisfy if it is to be included in a sample used to study redshift distributions. Seven QSOs for which results are available^{4,15} yield a well defined sample of 15 systems from a total sample window of $1.5 < z_{\text{abs}} < 3.3$. These systems are uniformly distributed in the redshift windows of their QSOs—showing no clumping at $z_{\text{abs}} \sim 2$.

QSO Ly α absorption lines

Ly α line samples are seen as particularly valuable in view of the widespread occurrence of redshift dependent selection effects in other data. The properties of these absorption lines in QSO spectra have been extensively discussed by Sargent *et al.*⁴, who conclude that the lines probably arise in intergalactic clouds. These lines are about an order of magnitude more numerous than the metal line systems.

Of fundamental importance is that, to first order, the detection of a Ly α cloud depends on only the intrinsic properties of

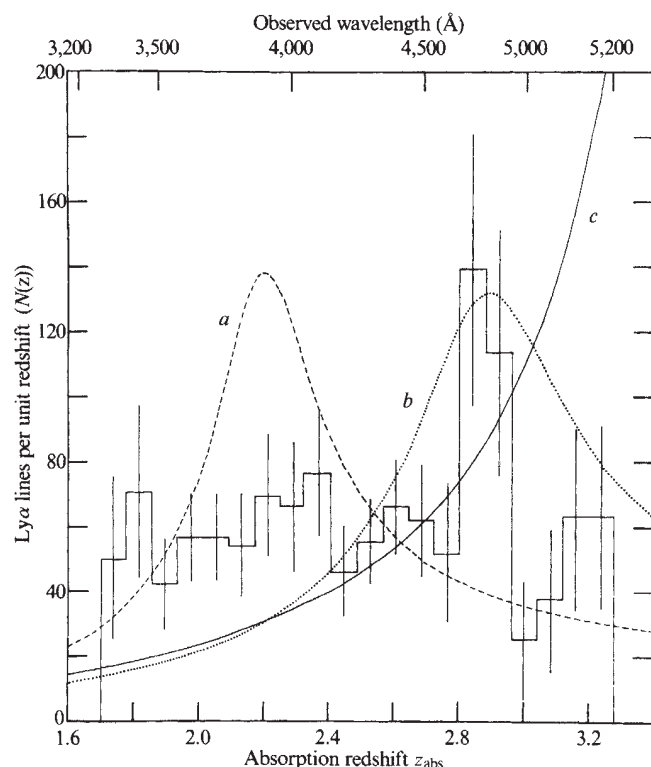


Fig. 2 The redshift distribution of Ly α lines. The statistical sample of 212 lines are shown in histogram form. The predicted $N_{\text{cos}}(z)$ distributions for three Lemaître models are also shown. It is assumed that the Ly α clouds do not evolve with redshift. Model *a* (dashed curve) has $\log_{10} \sigma_0 = -1.4$ and $\log_{10} \epsilon = -2.2$ giving a χ^2 probability of 9×10^{-6} for the observed distribution. Model *b* (dotted curve) for $\log \sigma_0 = -1.69$ and $\log \epsilon = -2.0$ was chosen to provide a comparison with the data peak. The χ^2 probability is 10^{-9} . Model *c* (solid curve) has $\log \sigma_0 = -1.9$ and $\log \epsilon = -2.2$ giving a peak at $z_p = 3.5$, outside the observed range, and a probability of 10^{-14} .

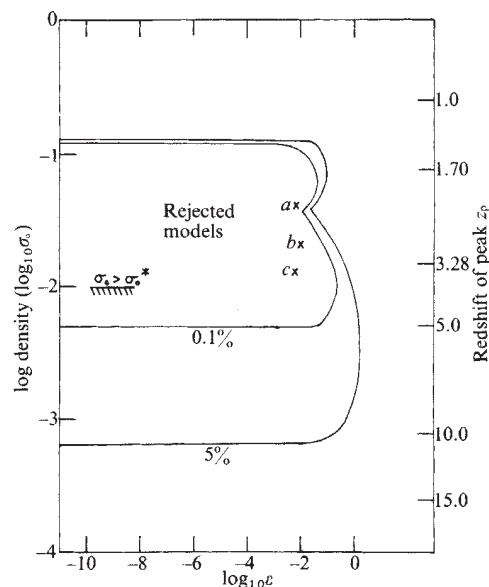


Fig. 3 Lemaître models which are rejected assuming that the Ly α clouds do not evolve. The z_p scale was obtained from the relation $\sigma_0^{-1} = (1 + z_p)^3 - 3(1 + z_p) + 2$, which only applies in the limit $\epsilon \rightarrow 0$. This scale gives z_p accurate to within 5% for models with $\log \epsilon < -1$ and 0.5% for $\log \epsilon < -2$. Models *a*, *b* and *c* from Fig. 2 are indicated. Percentage figures refer to the probability of falsely rejecting a model (that is, accepting that the observed distribution came, by chance, from the predicted one). Most of the models to the left centre of the 0.1% line are rejected with a negligible probability of false rejection. The small dip in the probability contours is purely the result of the geometry of fitting the predicted distributions to a data set of finite extent in redshift. The estimated lower limit on the density of the Universe⁸ is shown by σ_0^* . The upper density limit is extremely uncertain, although $\log \sigma_0 < +1$ does seem safe.

that cloud, and not on its redshift (but see below regarding possible evolution of the clouds). Four main observational biases affecting the possible detection of a Ly α line have been discussed in detail elsewhere^{4,15}. None of these closely inter-related difficulties is capable of either creating or hiding a Lemaître peak in the current data sample.

The redshift distribution of the statistical sample of 212 Ly α lines from seven QSOs^{4,15} is shown in Fig. 2. Data from more than one QSO (line of sight) contribute to all except for the first bin and the last six bins. The overall distribution is well represented by a constant density of 61 ± 4 lines per unit z ($1,216 \text{ Å}$ in the observed frame). There is, however, a prominent peak at $z \sim 2.9$. The two peaked bins span the wavelength range $4,627\text{--}4,819 \text{ Å}$ and contain 20 undoubtedly real lines (P. J. Young, personal communication), all from the line of sight towards PKS2126–158. As the expected mean content for any two bins is $\sim 9.6 \pm 0.6$ lines, the feature is of marginal significance. A preliminary analysis of observations of five other QSOs (R. F. Carswell, personal communication and ref. 16) shows that line density enhancement at $z \sim 2.9$ occurs only towards PKS2126–158, and so it cannot be associated with a Lemaître model peak. A possible physical origin for the enhancement is discussed below.

Rejection of Lemaître models

The test is straightforward, involving a numerical evaluation of the probability that the observed Ly α line distribution arose from the distribution predicted by various cosmological models. The 212 lines were divided into 20 bins each of width $\Delta z = 0.079$, spanning a total redshift range of $1.70\text{--}3.28$, and the predicted $N_{\text{cos}}(z)$ distribution was integrated numerically across each bin. The normalization constant N_0 was estimated and the

probability that the observed distribution came from the predicted one can be found using the χ^2 distribution. The predicted $N_{\text{cos}}(z)$ distributions for three particular models are shown in continuous form (before binning) in Fig. 2. Only models with $-1.83 < \log_{10} \sigma_0 < -1.13$ have peaks in the observed redshift range. Outlying models such as *c* ($\log_{10} \sigma_0 = -1.9$) are rejected because of a large variation of $N_{\text{cos}}(z)$ in the observed z range. Note that Lemaître peaks are too broad to fit the observed data peak. Narrower peaks can be obtained by decreasing ϵ , but these are then too high. The complete set of Lemaître models which are rejected on the assumption of a non-evolving population of Ly α lines is shown in Fig. 3.

Evolution of the Ly α clouds

The observed distribution of Ly α clouds is the product of the cosmologically specific redshift-path-length relation $N_{\text{cos}}(z)$, the cloud space density $\phi(z)$ and the mean cloud cross-section $\Sigma(z)$ at the critical H I column density. While the simplest assumption is that the observed flat distribution arises from the product of three slowly changing functions; we should consider the possibility that $N_{\text{cos}}(z)$, $\phi(z)$ and $\Sigma(z)$ all vary rapidly with redshift in a closely correlated manner such that their product gives the observed flat distribution.

Note that the peaks in $N_{\text{cos}}(z)$ arise because the Universe spends a long time at an approximately constant scale size $R = R_0/(1+z_s)$. There is ample time for a significant amount of evolution in $\phi(z)\Sigma(z)$ as z changes slightly on passing through a Lemaître peak.

A particularly simple model for the evolution of Ly α clouds has been presented Sargent *et al.*⁴ They propose that the clouds are pressure supported by an intergalactic medium which expands adiabatically with the Universe as a whole (see refs 17, 18 for alternative proposals). Adopting several simplifying

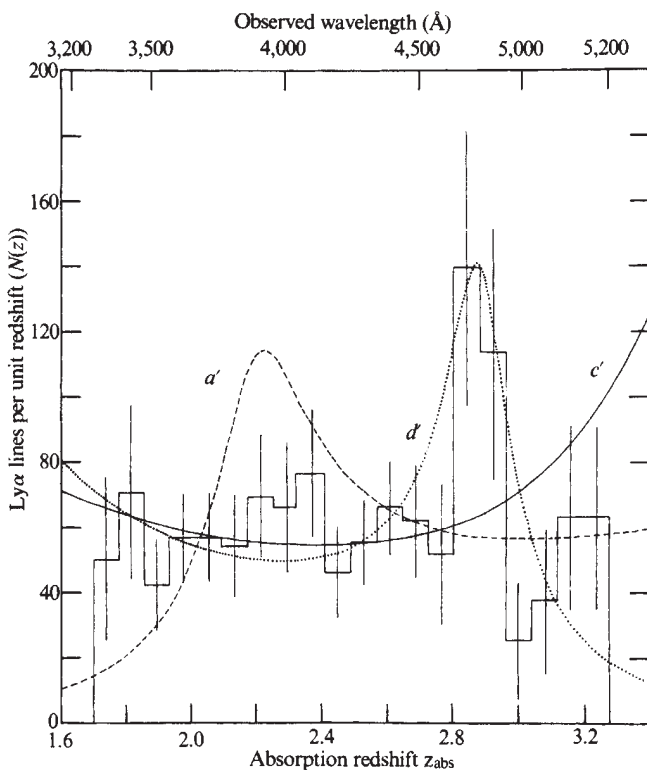


Fig. 4 The predicted distribution $N(z, G)$ of an evolving population of Ly α lines for three Lemaître models. The histogram shows the data, as in Fig. 2. Models *a'* (dashed curve) and *c'* (continuous curve) are the equivalents of models *a* and *c* in Fig. 2. Model *a'* has $G = 5.0$ and a χ^2 probability of 0.3%. Model *c'* has $G = -2.7$ and a probability of 28%. The third model *d'* has $\log \sigma_0 = -1.69$ and $\log \epsilon = -2.8$ giving $G = -5.0$ and a χ^2 probability of 27%.

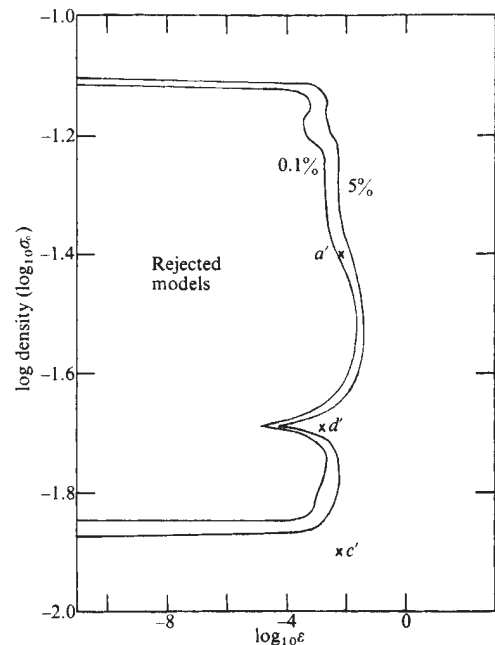


Fig. 5 The set of Lemaître models which are rejected on the assumption that the Ly α lines evolve as $(1+z)^G$. Models *a'*, *c'* and *d'* from Fig. 4 are indicated. Percentage figures refer to the probability of false rejection of a model. The small dip in the probability contours near $\log \sigma_0 \sim -1.2$ arises from the geometry of fitting the theoretical distributions to a data set of finite range in redshift.

assumptions, the resulting evolution is of the form $\phi(z)\Sigma(z) \propto (1+z)^G$, giving a predicted cloud distribution:

$$N(z, G) = N_0(1+z)^{2+G} |\text{RAD}|^{-1/2} \quad (4)$$

We cannot assign a unique value to the evolutionary parameter G without first accepting that some particular cosmological model is valid. Sargent *et al.* choose the standard Friedmann models ($\Lambda = 0$) and find that $G \sim -0.7$ is consistent with both the observed $N(z)$ as well as the line equivalent width distribution $n(W)$. In fact there is an error in the exponents of their equations (69)–(71); on correction we find that there is no longer any value of G which satisfies both $N(z)$ and $n(W)$. The obvious recovery is to drop the weakest of the many assumptions: that all the clouds have the same total mass. The result is a cloud mass distribution $n(M) \propto M^{-1}$ for $G \sim 0$ and $\sigma_0 = q_0 = \frac{1}{2}$; but G is still dependent on the *a priori* acceptance of a particular cosmological model (work in preparation).

In view of these uncertainties we treat G as a free parameter. We can then choose the values for G which make $N(z, G)$ in closest agreement with the observed Ly α distribution. The maximum likelihood method⁴ was used to estimate G . Figure 4 shows the $N(z, G)$ distribution for two of the models for which $N_{\text{cos}}(z)$ was shown in Fig. 2. A third model *d'* has the same σ_0 as *b*, but ϵ has been decreased to bring the $N(z, G)$ peak up to the height of the data peak. The complete set of models which are now rejected is shown in Fig. 5. The conspicuous dip in the probability contours at $\log_{10} \sigma_0 = -1.687$ arises because of the peak in the Ly α density at $z \sim 2.9$. As discussed above, it is highly unlikely that this feature is globally significant.

It is clear that the G parameter is able to counteract monotonic trends in $N_{\text{cos}}(z)$ and produce more acceptable fits to the observed distribution. It cannot remove peaks. Most of the models with $\log_{10} \epsilon < -2$ have $N_{\text{cos}}(z)$ distributions which vary rapidly across the observed z range. Some of these models are accepted in Fig. 5, but only if we are prepared to allow large absolute values for G ($4 \lesssim |G| < 9$). The metal line systems also seem to have an approximately flat $N(z)$ distribution. As it is

unlikely that both the Ly α clouds and the distinctly different metal line systems both evolve in the same manner, it is improbable that any model with $\log_{10} \sigma_0 < -1$ and $\log_{10} \epsilon < -2$ is valid.

We conclude by outlining four general constraints which evolution of $\phi(z)\Sigma(z)$ would have to satisfy if a Lemaître peak were to be successfully disguised. (1) $\phi(z)\Sigma(z)$ must decrease as $N_{\text{cos}}(z)$ increases on entering a quasistatic period, and then $\phi\Sigma$ must increase again as $N_{\text{cos}}(z)$ decreases on leaving the quasistatic period. Two evolutionary processes would be required to reproduce the observed flat distribution of $N(z) \propto \phi(z)\Sigma(z)N_{\text{cos}}(z)$. (2) Both evolutionary processes must be coupled to $N_{\text{cos}}(z)$ and hence to the global dynamics of the Universe because $N_{\text{cos}}(z) \propto H(z)^{-1}$. (3) $\phi\Sigma$ must return to close to its original value after the quasistatic period evolution—otherwise a sharp step would be seen in $N(z)$ at the redshift z_s . (4) Finally, if the evolution is to be able to disguise an arbitrarily sized peak, then the magnitude of the predicted change $\phi\Sigma$ must be related to the parameters which determine the peak height: ϵ or the duration of the quasistatic period. For small ϵ the required change in $\phi\Sigma$ during the quasistatic period is substantial: for $\log_{10} \epsilon < -3.3$, $N_{\text{cos}}(z_p)/N_{\text{cos}}(z_p + 0.5) \geq 10$.

It is clearly unlikely that more than one of these constraints can be met by any reasonable evolutionary model. Detailed models for the Ly α clouds, together with a better knowledge of the intergalactic medium and ionizing flux (see ref. 19) will be required to determine whether some of the weaker Lemaître peaks ($\log_{10} \epsilon > -2$) could have been disguised by evolution; but it does seem safe to assume that strong peaks cannot have been entirely hidden. Figure 5 may be regarded as a minimal set of rejected models, with Fig. 3 specifying the maximal set for the current data.

Superclustering of Ly α lines

The peak in the density of Ly α lines in the line of sight towards PKS2126–158 raises the important possibility that Ly α lines show weak clustering on a very large scale²⁰. Both the amplitude $(N - \bar{N})/\bar{N} \sim 1$ and the comoving size $\sim 60 h_{100}^{-1}$ Mpc for the feature towards PKS2126–158 are similar to values found for local superclusters of galaxies^{17,21}.

First, we must explain why the overall apparent incidence and/or mean amplitude of superclustering of Ly α lines is low, as shown by the near zero correlation function found by Sargent *et al.* Geometrical factors will be important if Ly α superclusters display the flat or chain-like morphology of galaxy superclusters^{17,22}, and it can then be shown that only the occasional passage of a line of sight down the long axis of a supercluster could produce even a marginally detectable line density enhancement.

A fundamental question raised by Silk (personal communication) is: why should Ly α clouds follow the distribution of galaxies by showing superclustering, when they apparently do not show small scale galaxy clustering? For an explanation we must first account for the surprising lack of small scale clustering. Ly α clouds in a region of space containing galaxies can be expected to have a distribution which is clustered, following the dominant gravitational influence of the galaxies. The sugges-

tion⁴ that Ly α clouds are modified or destroyed in the vicinity of galaxies must be regarded with caution, because it is possible that a negative rather than zero correlation function would arise. We then arrive at the Sargent *et al.* hypothesis in which most Ly α clouds exist in intergalactic regions, in which there are few galaxies and consequently minimal clustering. Indeed large volumes of space containing few galaxies have been identified in three-dimensional maps of galaxy positions²². An important consequence of this model is that there may still be a fraction $< \frac{1}{3}$ of all observed Ly α clouds which do exist amongst galaxies and follow the galaxy clustering. The non-clustered clouds would provide sufficient dilution to produce a net correlation function which is almost zero.

These simple considerations show that great care will be required in the interpretation of Ly α cloud clustering. Jones²³ has stressed that it is difficult to think of observations which will help to distinguish between the rival 'gravitational instability' and 'pancake' scenarios for the formation of galaxies. It can be expected that future studies of the evolution of clustering of absorption lines out to $z \sim 3$ will be of great relevance to this topic. For example, the existence of superclusters with density enhancements as large as ~ 1 at the early epoch of $z \sim 2.9$ would favour a 'pancake' scenario.

Conclusions

We have shown that, subject to the reasonable assumptions that the Ly α lines are at distances given by their redshifts^{4,24–26} and that they do not evolve in a manner significantly more complex than a function of form $(1+z)^G$ over the redshift range 1.7–3.3, a wide range of Lemaître modes can be rejected because of the lack of peaks in the observed distribution of Ly α lines.

This analysis is now being extended to models with higher densities in the range $0.1 < \sigma_0 < 10$ using a variety of data.

The distribution of an unbiased but small sample of narrow metal absorption line systems seen in QSO spectra shows no evidence for strong peaks in their redshift distribution. Clearly an earlier result showing a peak at $z_{\text{abs}} \sim 2$ suffered from strong selection effects. The flat distribution supports the rejection of the Lemaître models as it is unlikely that the Ly α clouds and metal line clouds both evolve in the same manner, which would have to be closely correlated with the dynamics of the Universe as a whole, if the Lemaître model $N(z)$ peaks are to be extensively suppressed.

Evidence against the Lemaître models, including the $N(z)$ peak test used above, has been discussed elsewhere^{5,6}. Tinsley³ has pointed out that many of the other tests are subject to various difficulties and it now seems highly unlikely that any of them could be as strong or as direct as the $N(z)$ test using Ly α clouds for the low density (σ_0) Lemaître models shown in Fig. 3.

Further details of the present work here will be published elsewhere.

I thank A. Boksenberg, W. L. W. Sargent, P. J. Young, M. R. Rees and R. S. Ellis for many helpful discussions, and R. F. Carswell for permission to use unpublished data. J. Silk offered many helpful suggestions. Financial support from the SRC is acknowledged.

Received 14 January; accepted 24 April 1981.

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Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*)

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Homozygous diploid zebra fish have been produced on a large scale by the application of simple physical treatments. Clones of homozygous fish have been produced from individual homozygotes. These clones and associated genetic methods will facilitate genetic analyses of this vertebrate.

HETEROZYGOSITY in diploid eukaryotes often makes genetic studies cumbersome. Here we describe the ready production of homozygotes in a vertebrate. Our methods have been developed to allow the eventual dissection of neuronal development by the use of mutant strains^{1,2}, but are also likely to find use in a wider range of applications.

We have chosen the tropical freshwater cyprinid zebra fish *Brachydanio rerio*^{3,4} for several desirable attributes⁵: the generation time is only 3–4 months; at weekly intervals mature females lay several hundred eggs which develop rapidly and synchronously outside the mother; the fish are small (3 cm), hardy and easy to care for. Large-scale screening for mutants is possible because free-swimming 7-day-old fish exhibit many behavioural and morphological traits of the parent but are only a few millimetres long. Because development is normal between 25 and 31 °C (see also ref. 6) it should be possible to isolate temperature-sensitive mutants.

To produce homozygotes, eggs are activated by genetically impotent sperm. The haploid maternal set of chromosomes is allowed to replicate once, and partition of the duplicated chromosomes into two cells is prevented by hydrostatic pressure or heat shock. A cell with two identical sets of chromosomes is thereby produced. Our procedure is similar in principle to that described recently for the production of individual homozygous mice^{7,8}.

The generation of large numbers of homozygous diploids makes it possible to detect rare newly arisen recessive muta-

tions. Linkage analysis is facilitated because the genotypes of gametes produced by a female are directly reflected by the phenotypes of her homozygous progeny. Large clones of diploid homozygous fish with identical genetic constitutions can be generated from individual homozygous females.

Production of homozygous diploids

The union of a sperm with an egg ordinarily serves both to activate it (initiate cleavage divisions) and to restore a diploid set of heterozygous chromosomes. We eliminated heterozygosity by activating zebra fish eggs with UV light-treated⁹ sperm (UV sperm). These eggs develop into inviable haploid embryos with bent tails and vacuolated body cavities, as has been described for various lower vertebrates^{10–12}. About 3×10^{-4} normal embryos appear; one such embryo whose karyotype has been examined was diploid and may have arisen through nondisjunction.

To generate viable embryos, haploid eggs were converted to diploids by either hydrostatic pressure or heat shock. In the late pressure treatment (LP), eggs activated by UV sperm are exposed to a combined pulse of ether and hydrostatic pressure at the first mitotic division (Fig. 1). Hydrostatic pressure dissociates microtubule subunits¹³ and prevents cell division¹⁴; it has been used to inhibit the second meiotic division in frogs¹⁵. Ether inhibits cytokinesis in sea urchin eggs¹⁶. The establishment of the optimum treatment will be described elsewhere.

Females which produced grossly abnormal haploids (probably because of deleterious genes) were eliminated from our starting

Table 1 Survival of progeny from eggs treated to produce homozygotes

Population*	Treatment	No. of mothers	Fraction of fertile eggs which develop into normal-appearing 24-h embryos	Fraction of normal-appearing embryos which survive to: 9 Days with swim bladder† Adulthood‡	Total no. of surviving progeny counted as adults or at 9 days with swim bladders
Starting	LP	90	0.29		874
Selected I (8 different sets)¶	LP	49	0.20	0.73	831
Selected II (Set 1)	LP	4	0.28	0.69	146
(Set 2)	LP	17	0.09	0.46	73
Selected III (Set C29 × C32)	HS	28	0.33	0.61	501#
Clone C29 (Group III homozygote)	HS	18	0.25	0.38	85
Clone C29	EP	8	0.47	0.68	421
Clone C32 (Group III homozygote)	EP	8	0.51	0.81	494

* See Fig. 2.

† The fraction of embryos which develop swim bladders is influenced by the nutritional regime and general well-being of the mothers, especially for homozygous fish. Fish aged ≥ 1 month are fed twice a day with a rotation of: Tetra-min staple food (Tetra), Oregon test diet²⁸ and Oregon moist pellets (Bioproducts), liver-spinach pâté²⁹ and brine shrimp (*Artemia*) nauplii (eggs from Aquarium Products). If adult brine shrimps are available they can be substituted for the Oregon test diet, moist pellets and brine shrimp nauplii. Fish aged 4–14 days are fed paramecia and 9–30 day old fish are fed brine shrimp nauplii.

‡ Ordinarily, about 90% of the 9-day-old fish with swim bladders develop into adults.

§ Survival is for fish that have reached an age of 3 months.

¶ Each set is the product of a cross of two homozygous individuals [or in the case of selected population III (set C29 × C32), the product of crosses between two clones]. Different sets have different homozygous parents.

|| Survival is for fish that have reached an age of 6 months.

Only a fraction of the progeny of each mother are reported here because some portions were subjected to other treatments.

Pressure pulse

Ether pulse

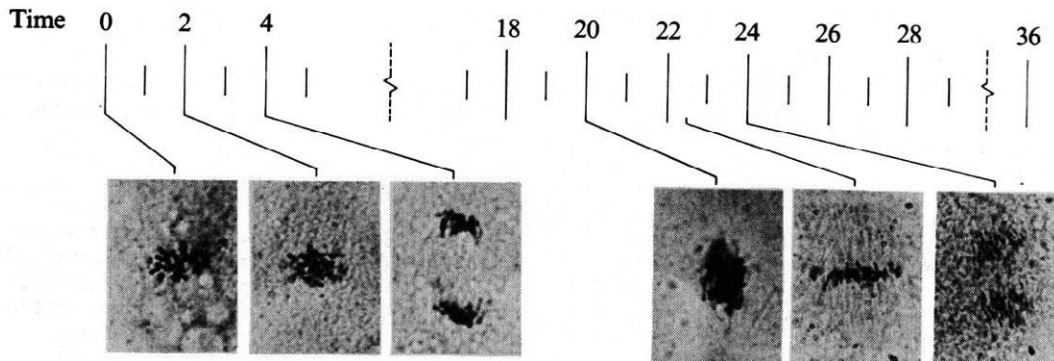


Fig. 1 Late pressure (LP) treatment. Groups of females were maintained at about 27 °C with a twofold excess of males, in a 14 h light–10 h dark cycle, at about one fish per l. To collect eggs, females were segregated from males about 17 h before the beginning of a light cycle. Shortly before fertilization was due, males were anaesthetized in 0.2 mg ml⁻¹ ethyl *m*-aminobenzoate methanesulphonate (Finquel, Ayerst) [brought to pH 7.0 with Tris(hydroxymethyl)aminomethane], and sperm were expelled by gentle pressure with smooth forceps, and taken up into a capillary pipette. Sperm from four or five males were pooled in 0.4 ml of cold Hanks' saline to give a concentration of about 10⁸ sperm per ml. Sperm in a watch glass were exposed to UV light from a 43-cm (Sylvania) germicidal tube at a distance of 29 cm for 2 min. For about 90 min after the beginning of the light cycle, eggs were expelled from anaesthetized females in 3.5-cm plastic Petri plates (Lux brand, as some batches of another brand proved toxic to zebra fish embryos) by gentle finger pressure. Eggs were activated by addition of 0.05 ml of sperm suspension and 0.5 ml water [glass-distilled and supplemented with 1 g l⁻¹ sea salts (Instant Ocean, Aquarium Systems)] at 28.5 °C, followed by further water after about 30 s. Activated eggs were incubated at about 28.5 °C. Eggs were transferred to glass vials and at 17 min after activation water was removed and replaced by 2% v/v aqueous ether. The vials (filled to the brim) were closed by a thin sheet-rubber diaphragm, held in place with the vial's snap-on plastic cap into which a hole had been cut. The vial(s) were placed into a French press, and at 22.5 min the pressure in the chamber was raised to 8,000 p.s.i. with a hydraulic press. (Pressure gauge dials on many hydraulic presses are calibrated to read pressures exerted on a 2-inch diameter piston, and readings must be converted to p.s.i.) At 28 min the pressure was reduced to ambient over 1 min and at 36 min the ether solution was gradually replaced by (salt-supplemented) water. The procedure was carried out at about 28.5 °C. Several hours after treatment, eggs were examined under a low-power microscope and any which appeared infertile or grossly abnormal were removed; the latter were incubated in separate dishes. The fraction of fertile eggs was determined in an aliquot which had not been exposed to LP; ordinarily, more than 80% of the eggs were fertile. The meiotic and mitotic figures shown under the time scale were from Feulgen-stained squashes of entire eggs. The first three inset figures show the second meiotic division and the next three the first mitotic division. The times indicated for the various stages are based on observations of a series of squashes. To obtain karyotypes 24–48 h (post-activation) embryos were removed from their shells and transferred to 10⁻² M colchicine (Sigma) for 90 min. Their yolk sacs were then dissected away and the embryos transferred to 0.04 M sodium citrate and incubated for 7.5 min at 21 °C and for a further 7.5 min at about 4 °C. They were then transferred to a 3:1 (v/v) solution of methanol/acetic acid and stored at room temperature for about 30 min and then at about -20 °C. To make spreads, embryos were minced in a few drops of 50% acetic acid for 2 min, and then droplets of the suspension were repeatedly and rapidly deposited onto and removed from slides at 50 °C (ref. 30). After drying, the slides were incubated in Giemsa's stain, washed in water, dried and examined. For a mature fish, karyotypes from regenerating fins were prepared in a similar manner, except that the regenerating portions of fins were clipped³¹ and incubated in medium containing 1.3 × 10⁻⁵ M colchicine, 10% fetal calf serum and 2 × 10⁻³ M glutamine. About eight spreads were counted for each fish.

population. About 29% of the LP-treated eggs from the starting population developed into embryos which appeared indistinguishable from normal diploid embryos at 24 h after fertilization (Table 1). At 24 h we examined karyotypes of 22 developed, but otherwise unselected embryos; 20 were diploid (2N = 50 chromosomes¹⁷), 1 was haploid and 1 aneuploid. The latter two embryos appeared abnormal and were not expected to survive. As the genetic contribution of sperm was eliminated by UV light, we expect that the diploid fish created by LP have two identical sets of maternally derived chromosomes and are thus homozygous.

About 20% of the normal-appearing 24-h LP embryos from the starting population survived to maturity (Table 1). The losses could be due to deleterious genes or to the destructiveness of the LP treatment. To select for populations of hardy fish we carried out the procedure outlined in Fig. 2. We crossed surviving group I homozygous fish which appeared relatively vigorous, long-lived and fecund. For crosses we used either spontaneously occurring homozygous males, or ones produced by the hormone treatments described below. The heterozygous offspring were raised to maturity. The LP treatment applied to eggs of these selected population I heterozygous fish yielded a new generation of homozygous fish, designated as group II in Fig. 2. As 68% of the normal-appearing group II homozygotes survived to maturity (Table 1), the enhanced survival is probably due to a decrease in the number of deleterious genes. Most mature LP fish are diploids: of 31 group II homozygotes, 30 had a modal number of 50 chromosomes in metaphase spreads obtained from colchicine-treated regenerating tail fins and one fish exhi-

bited approximately equal numbers of spreads with 50 and 52 chromosomes. The most vigorous individuals among the group II homozygotes were crossed, and from the selected population II set produced by one pair of fish, the group III homozygotes were obtained by LP. From these fish we derived clones (for example C29 and C32) as described below.

In the heat shock (HS) treatment for generating diploids, eggs activated by UV sperm were incubated at an elevated temperature (for example, 41.4 °C) for a 2-min interval about 10 min before metaphase (at 13 min post-activation at ~28.5 °C). Heat shocks at analogous times inhibit a subsequent cycle of mitosis in *Physarum*¹⁸, and have previously been used to inhibit the second meiotic division in lower vertebrates^{19–21} and to produce tetraploid newts²².

For many homozygous fish (and clones, see below) HS is far more effective than LP and for many populations 10–20% of HS-treated fertile eggs develop into adults (for example clone 29 and selected population III; Table 1). The karyotypes of 12 normal-appearing HS embryos were examined; all were diploids. The optimal temperature and the efficiency of the HS treatment depend on the genotype of the mother, as will be described elsewhere. For many strains the relative simplicity of the HS treatment makes it the method of choice.

The experiments described in the next section were designed to measure the extent of heterozygosity due to the occasional contribution of genes by UV sperm or to chromosome nondisjunction. In addition, we introduce some convenient genetic markers and illustrate analyses of gene-centromere distance and segregation.

Genetic contribution by UV sperm

The genetic contribution of the UV sperm was measured using a spontaneous recessive mutation (designated b1) in the *gol-1* (golden) gene. Fish that are *gol+ / gol+* or *gol-1 / gol+* become pigmented within 48 h of fertilization (Fig. 3a) but the *gol-1 / gol-1* mutants lack pigmented melanocytes up to 76 h (Fig. 3b). The *gol-1* gene segregates as a typical mendelian recessive; 50% of the LP progeny of *gol-1 / gol+* heterozygotes are pigmented and 50% are pigmentless; crosses between heterozygous fish yield 25% progeny with the golden phenotype. Sperm of *gol+ / gol+* males were exposed to 10^3 erg mm⁻² UV light and used to activate eggs of *gol-1 / gol-1* females. Of more than 600 embryos, none was observed to have any pigment. As the UV sperm used for producing homozygous fish are exposed to a dose that is four times as high (4×10^3 erg mm⁻²), heterozygosity due to the genetic contribution of the sperm seems unlikely.

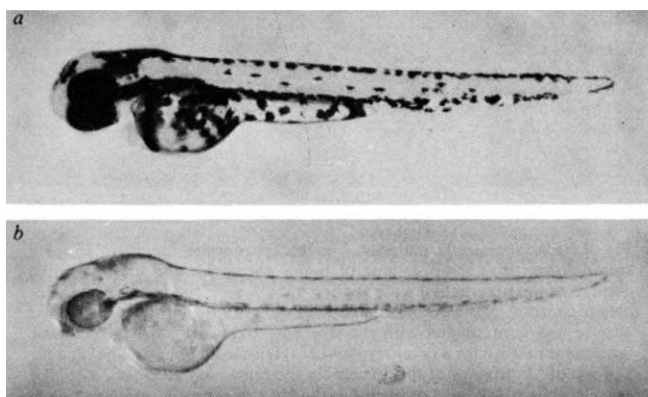


Fig. 3 Pigment differences between normal and mutant (*gol-1*) fish. *a*, A 3-day-old *gol+ / gol+* zebra fish; *b*, a 3-day-old *gol-1 / gol-1* zebra fish. These fish are about 3.8 mm long.

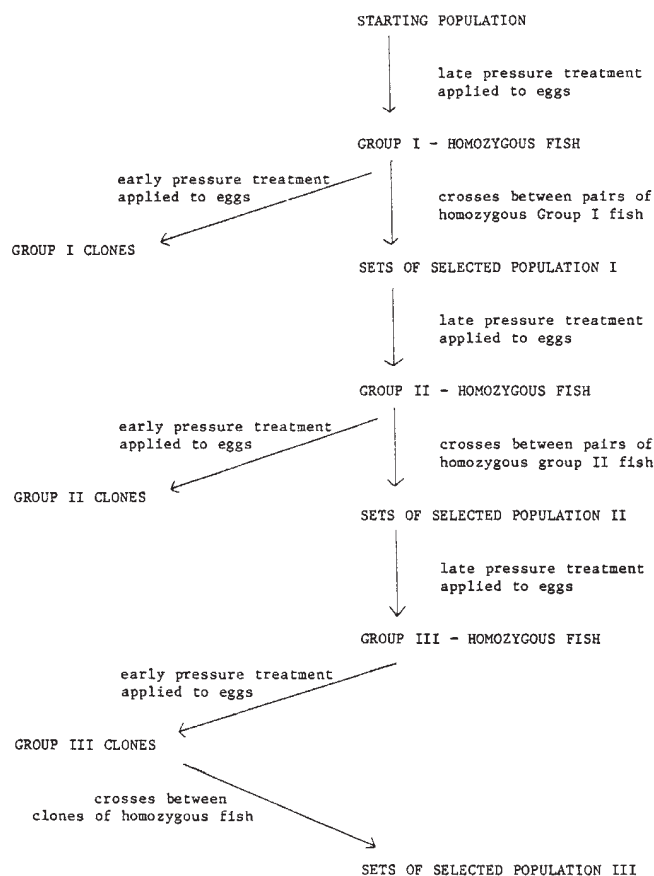


Fig. 2 Schematic diagram of the production of homozygous fish and clones.

Nondisjunction during meiosis I

Nondisjunction of the chromosomes of a heterozygous mother during meiosis will result in heterozygosity among her LP progeny. To estimate the magnitude of this contribution, we used electrophoretically different allozymes²³ which produce distinguishable phenotypes in heterozygotes and homozygotes.

Esterases have been described in zebra fish²⁴; we found a set of three electrophoretically different esterase bands (designated as 3, 4 and 5) which seem to be specified by three alleles of a single gene we call *est-3*. On electrophoresis of individual fish extracts, one or two but never all three bands were observed. Phenotypes of homozygous and heterozygous fish will be described as 3, 4 or 5, and 3/4, 3/5 or 4/5, respectively. Another

band of esterase activity (band 2) seems to be specified by a gene closely linked to *est-3*, as will be described elsewhere.

To detect nondisjunction, we measured the frequency of heterozygotes among the LP progeny of heterozygous mothers who were formed by crossing pairs of homozygotes and exhibited 3/4, 3/5 or 4/5 band patterns. As shown in Table 2 and Fig. 4, no heterozygotes were observed among the 476 LP progeny examined. These results show that the three bands are specified by alleles or by very closely linked genes (we demonstrate below that recombination does occur in zebra fish).

Nondisjunction at the first meiotic division would result in heterozygosity in almost every case in which the unseparated chromosomes are retained in the egg. As no heterozygotes were observed among 476 progeny, heterozygosity for markers on the *est-3* chromosome occurs with a frequency of less than 1% (considering 95% confidence limits) among mature fish. Nondisjunction could, of course, be more frequent but result in lethality or it could be more frequent for other chromosomes.

Nondisjunction during meiosis II

Nondisjunction during the second meiotic division does not necessarily result in heterozygosity: the sister chromatids remaining in the egg before the second meiotic division will be identical except for regions separated from their original centromere by genetic recombination. Nondisjunction can be detected only in eggs that contain sister chromatids which are heterozygous.

To measure the frequency of heterozygous sister chromatids, eggs of 3/4, 3/5 and 4/5 mothers were subjected to a treatment which inhibits the second meiotic division^{9,25,26}; in zebra fish, as in many other vertebrates, this division occurs after fertilization. Exposure of zebra fish eggs activated by UV sperm to a pulse of 8,000 p.s.i. hydrostatic pressure for 1.5–6 min post-activation (the early pressure or EP treatment) effectively inhibits the second meiotic division and results in >30% viable diploid embryos (Table 1). These fish contain a diploid complement of chromosomes because the sister chromatids are retained in the

Table 2 Segregation of band pattern phenotypes among LP progeny of heterozygous parents

Mothers with bands	No. of LP progeny with bands			Total
3 and 4	(3)	(4)	(3 and 4)	Total
	72	59	0	131
3 and 5	(3)	(5)	(3 and 5)	Total
	73	58	0	131
4 and 5	(4)	(5)	(4 and 5)	Total
	105	109	0	214
Total no. of LP progeny:				476

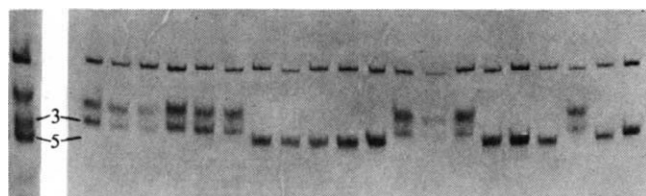


Fig. 4 Electrophoretic patterns. Fins of appropriate fish were clipped, stored frozen in a small volume of buffer overnight, and after thawing, aliquots of the supernatant were applied to 10% acrylamide slab gels. After electrophoresis, gels were incubated with the esterase substrate α -naphthyl acetate and then stained with the dye Fast Blue RR³². Electrophoretic patterns are shown for a heterozygous (3/5) female (in the first lane) and for a group of LP progeny of this female (in the remaining 20 lanes). Note the presence of either band 3 or band 5 in the lanes of progeny extracts. A gene specifying the band 2 esterase co-segregates with the *est-3* gene.

egg nucleus. Among the progeny fish which developed from these sets of EP-treated eggs, 14% (87/626) exhibited two of the appropriate bands and were thus heterozygous. These results and other gene-centromere distances will be described more fully elsewhere.

Nondisjunction at the second meiotic division could thus have been detected in only 14% (or about 67) of the 476 LP progeny described in Table 2. The absence of heterozygotes suggests that fewer than 6% of mature fish (based on 0/67, with 95% confidence limits) develop from eggs which have experienced second meiotic division nondisjunction of the chromosome bearing the *est-3* gene.

Clones of homozygous diploids

For a ready interpretation of the consequences of particular mutations, standard and mutant strains ideally should differ at only one site. We generated clones of homozygous fish by EP or HS treatment of eggs of homozygous mothers. The many fish in a particular clone are all derived from the same single cell (the UV sperm-activated egg from which the clone's mother developed) without sexually contributive processes.

Clones have also been propagated by matings of 'hormone-created' males²⁷ and their normal sisters. Ordinarily, more than 95% of the fish in the C29 clone are females. Exposure of C29 embryos for 2 weeks, beginning 1 day after fertilization, to 1.7×10^{-7} M 17 α -methyltestosterone (Sigma) resulted in the production of more than 75% males (defined by production of functional sperm) and few, if any, females.

The clones derived from various homozygous individuals of selected population II (Fig. 2) differed with respect to viability, longevity and fecundity. Some clones contained a small fraction of individuals with characteristic physiological abnormalities, probably because of the presence of recessive, deleterious, low-penetrance alleles inherited from the starting population. Such clones as C29 and C32 (group III) are similar to the starting

population with respect to desirable properties. Indeed, successive generations of these clones have been in routine use in our laboratory for large-scale experiments. However, although suitable for routine use, C29 and C32 fish seem more subject to stress, exhibit more stringent dietary requirements and produce a larger fraction of poorly developing eggs than do wild-type fish.

More vigorous hybrid fish were produced by crossing two clones of homozygous fish (for example, C29 and C32 to produce selected population III). These hybrids are satisfactory for the isolation of mutants because they are free of lethal genes. As several of the matings described in Fig. 2 are between siblings, the progeny of selected population III are expected to be homozygous at about 75% of the genome.

A surprising observation is that some clones (derived by HS or EP from single homozygous females) are predominantly male. Subsequent generations of such clones also contain a high frequency of males. The genetic basis of sex determination in zebra fish is unknown.

Conclusions

The large-scale production of homozygous zebra fish has already enabled us to isolate mutants, to measure frequencies of induced recessive lethals, to recognize fish heterozygous for recessive lethals and to measure recombination frequencies.

At present, heat shock seems to be the simplest method for producing homozygotes, although for some clones we have not been able to find productive temperature regimes.

The genetic methods we describe may be applicable to fish such as trout, salmon and catfish, which serve as human food. Treatments which inhibit the second meiotic division have been used successfully for such fish^{22,23}, and G. Thorgaard (personal communication) has been able to inhibit the first cleavage division in a fraction of treated trout eggs with heat shocks. The generation of clones may facilitate isolation of stocks with desired characteristics. In analogy with common agricultural practice, it may be possible to maintain clones for the generation of high-yield hybrid lines for harvest. If such endeavours are contemplated, it is crucial to keep in mind the need for preserving large fractions of the natural populations of any given species in their natural environment. As clones such as we have developed contain only a small fraction of the gene pool of the natural population, a large-scale displacement of natural populations by specific clones could easily lead to losses of entire species due to unexpected selective forces.

We thank Harriet V. Kuhnlein for her assistance at the beginning of this project, S. Chakrabarti and J. Golin for providing unpublished data, our colleagues at the Institute of Molecular Biology and Department of Biology for encouragement, Ira Herskowitz for enthusiastic interest and suggestions concerning this manuscript, R. O. Sinnhuber and J. D. Hendricks for Oregon moist pellets and test diet, Dr G. Thorgaard for advice in the preparation of karyotypes, and Kathleen Teichman and Julie Dunn for secretarial help. This work was supported in part by NSF grant P3B 3128 and NIH grant R01 GM 22731.

Received 25 August 1980; accepted 13 March 1981.

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DNA-dependent RNA polymerase II of plant origin transcribes viroid RNA into full-length copies

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DNA-dependent RNA polymerase II purified from healthy plant tissue is capable of synthesizing linear (–)-viroid RNA copies of full length from (+)-viroid RNA templates in vitro. Together with the specific α -amanitin sensitivity of viroid replication observed in vivo, these findings suggest that viroids replicate by an entirely novel mechanism in which infecting viroid RNA molecules are copied by the host enzyme which is normally responsible for the synthesis of nuclear precursors to messenger RNA.

VIROIDS, the smallest known agents of infectious diseases, are responsible for several economically important diseases of higher plants. They are single-stranded covalently closed circular RNA molecules consisting of about 360 nucleotides, which form a highly base-paired rod-like structure with unique properties^{1–3}. Thus, the 359 nucleotides of the potato spindle tuber viroid (PSTV)² are arranged in 26 double-stranded segments separated by 25 regions of unpaired bases embodied in single-stranded internal loops; there is a loop at each end of the rod-like molecule. These features provide the viroid RNA molecule with structural, thermodynamic and kinetic properties very similar to those of a double-stranded DNA molecule of the same molecular weight and G · C content³.

The mechanism of viroid replication has long been puzzling. The limited coding capacity of the viroid genome argued against the synthesis of a viroid-specific replicase in the infected cell and, indeed, viroid-specific polypeptides have not been found. Thus, viroid replication would depend on the host nucleic acid-synthesizing machinery. Nor do viroids exist in a DNA form integrated into the host genome and from which new

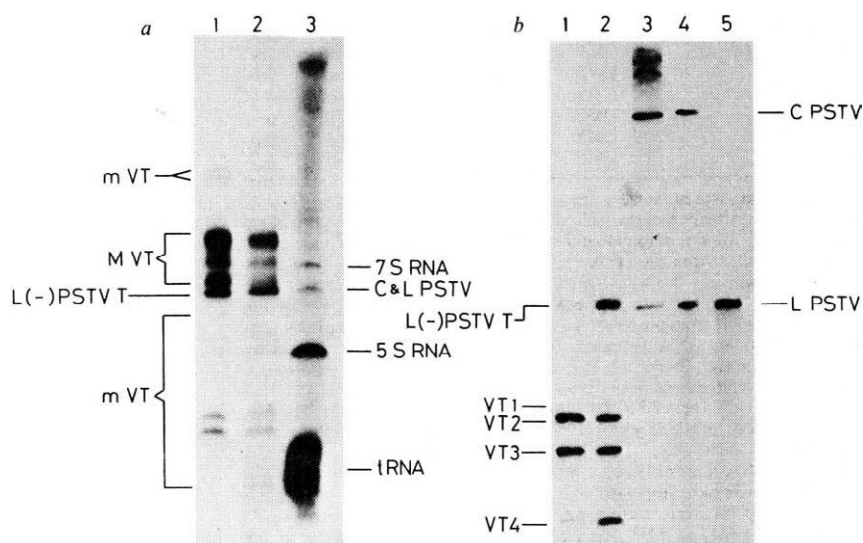
progeny viroid could be transcribed^{4–6}, since stringent molecular hybridization has shown that this is not the case^{7,8,30}.

Our finding that viroid synthesis is inhibited *in vivo* by an intracellular concentration of 10^{-8} M α -amanitin strongly suggested that the nuclear DNA-dependent RNA polymerase II is involved in the initial stages of viroid replication⁹. We now report that this enzyme, purified from healthy viroid host plant tomato tissue and from wheat germ, is able to synthesize full-length viroid RNA copies *in vitro* with viroid (+)RNA as template. (The isolated and infectious viroid RNA is arbitrarily called (+) viroid RNA.)

Template specificity of RNA polymerase II

DNA-dependent RNA polymerase II was isolated from either callus or green leaf tissue of tomato (*Lycopersicon peruvianum* (L.) Mill.), an excellent host for different viroid 'species'. The purification followed essentially the procedure developed by Jendrisak and Burgess¹⁰ for wheat-germ polymerase II (Table 1). With both enzymes, standard single-stranded DNA templates are transcribed less efficiently than double-stranded

Fig. 1 Analysis by polyacrylamide gel electrophoresis (PAGE) and autoradiography of ³²P-labelled RNAs transcribed from viroid (PSTV) RNA by DNA-dependent RNA polymerase II from tomato tissue and wheat germ. Incubation mixtures for RNA synthesis contained at a final volume of 500 μ l the cofactors described in Table 1 legend as well as 500 ng of purified PSTV RNA, 1.5 μ g of tomato and wheat-germ RNA polymerase II, respectively, and 100 μ Ci of each of the [α -³²P]-labelled ribonucleoside triphosphate (specific activity 400 Ci mmol⁻¹). The reaction was stopped after 4 h at 37 °C by adding EDTA to 0.01 M. After the addition of 100 μ g of yeast carrier tRNA and phenol extraction, the mixtures were chromatographed on 20-ml columns of Sephadex G-50 in (TNE) buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA). The peak fractions of radioactively labelled RNA in the exclusion volume of the columns were pooled, RNA precipitated with 3 vol of ethanol and collected for PAGE by centrifugation. Pellets were redissolved in 20 μ l of 89 mM and 22.3 mM TEB buffer, respectively, containing 8 M urea and xylene cyanol FF and bromophenol blue as dye markers. Marker RNAs were ³²P-labelled, LiCl-soluble RNA from PSTV-producing potato callus isolated according to ref. 27 and ¹²⁵I-labelled PSTV prepared according to the method of Commerford²⁹. **a**, PAGE on a 5% polyacrylamide slab gel (140 × 190 × 1.5 mm) at 10 °C and in the presence of high salt (89 mM) TEB buffer and 8 M urea. 1, Transcriptional products of wheat-germ RNA polymerase II. 2, Transcriptional products of tomato RNA polymerase II. 3, ³²P-labelled cellular RNA from PSTV-infected tomato callus²⁷. MVT, major viroid (PSTV) transcripts; mVT, minor viroid (PSTV) transcripts; L (–) PSTV T, linear viroid (PSTV) transcript. **b**, PAGE on a 5% polyacrylamide gel at 40 °C in the presence of low salt (22.3 mM) TEB buffer and 8 M urea. 1, Transcriptional products of wheat-germ enzyme. 2, Transcriptional products of tomato enzyme. 3, ³²P-labelled cellular RNA. 4, Circular (C PSTV) and linear (L PSTV) ¹²⁵I-labelled PSTV RNA. 5, Linear ¹²⁵I-labelled PSTV RNA. VT1–VT4, viroid-(PSTV) RNA-directed RNA transcripts.



DNA templates but the transcription of single-stranded DNA templates is considerably improved in the presence of oligo-RNA primers (Table 1).

Synthetic and natural RNAs also served as templates for transcription, thus confirming previous results on the same enzyme from pea and cauliflower¹¹⁻¹³. In our case, tobacco mosaic virus (TMV) RNA and soluble RNA (5S RNA and tRNA) from yeast were relatively poor templates, whereas the synthetic template/primer poly(rA)/oligo(rU) is transcribed with high efficiency. Most surprisingly, however, of all natural RNA templates tested, viroid RNA is transcribed with the highest efficiency by DNA-dependent RNA polymerase II from tomato and wheat germ (Table 1). When PSTV, citrus exocortis viroid (CEV) and chrysanthemum stunt viroid (CSV) were compared with respect to their template efficiency, PSTV RNA was transcribed best by the tomato polymerase II. PSTV was propagated in and isolated from tomato (*Lycopersicon esculentum* Mill. cv. 'Rentita') whereas for CEV *Gynura aurantiaca* DC and for CSV *Chrysanthemum morifolium* Ramat. cv. 'Yellow Delaware' were used as host plants. These three viroids also showed marked differences of their oligonucleotide fingerprints and hence their primary sequence¹⁴. Although overall transcription was about the same when circular and linear viroid

Table 1 Transcription activity of different DNA and RNA templates for DNA-dependent RNA polymerase II from tomato tissue and wheat germ

a DNA templates	³ H-NMP	Incorporation			
		Tomato polymerase		Wheat-germ polymerase	
		Counts per 30 min	%	Counts per 30 min	%
Poly(dA)	UMP	2,425	13.2	1,583	8.3
Poly(dA) · poly(dT)	UMP	6,175	33.7	11,514	62.3
Poly(dA-dT)	UMP	18,312	100	18,470	100
Poly(dG-dC)	CMP	15,240	83.2	6,488	35.1
Poly(dA) · oligo(rU)	UMP	30,160	164.7	19,225	104.2
Tomato DNA	UMP	4,210	23.0	1,849	10.0
Salmon sperm DNA	UMP	5,305	28.9	2,030	11.0
Calf thymus DNA	UMP	5,460	29.8	2,476	13.4
b RNA templates		Counts per 60 min		Counts per 60 min	
			%		%
Poly(rA)	UMP	560	4.4	257	1.4
Poly(rA) · poly(rU)	UMP	256	2.0	316	1.7
Poly(rA-U)	UMP	366	2.9	739	4.0
Poly(rA) · oligo(rU)	UMP	12,630	100	18,287	100
TMV RNA	UMP	609	4.8	640	3.5
Yeast soluble RNA	UMP	220	1.7	358	1.9
PSTV RNA	UMP	1,658	13.1	1,366	7.5
CEV RNA	UMP	1,038	8.2	1,108	6.1
CSV RNA	UMP	1,196	9.5	1,220	6.7
L PSTV RNA	UMP	1,315	10.4	1,201	6.6
C PSTV RNA	UMP	1,463	11.6	1,135	6.2

RNA polymerase was isolated by precipitation from the clarified crude extract by Polymin P, re-extraction of the pellet by high salt buffer, ammonium sulphate precipitation and column chromatography on DEAE-cellulose. Tomato polymerase II was eluted from the DEAE column with 0.4 M ammonium sulphate buffer, pH 7.9, whereas the wheat-germ enzyme elutes at a 0.25 M salt step. According to its subunit pattern in SDS-containing polyacrylamide gels, the tomato enzyme differs from the wheat-germ enzyme, but it behaves similarly with respect to substrates, DNA templates, metal ions and specific inhibition by 10⁻⁸ M α -amanitin (ref. 28 and H.-R.R. & H.L.S., manuscript in preparation). Assays for RNA synthesis were performed at 37 °C in a total volume of 250 μ l containing Tris-HCl, pH 7.35 (10 mM), MgCl₂ (6 mM), MnCl₂ (1 mM), (NH₄)₂SO₄ (40 mM), ethylene glycol (10%), unlabelled nucleoside triphosphates (NTPs) (400 μ M each), 25 μ Ci ³H-NTPs (specific activity 52 Ci mmol⁻¹) as indicated. For DNA-directed RNA synthesis (a) the NTP concentration of the ³H-labelled NTP was raised to 40 μ M by addition of the corresponding unlabelled NTP. DNA template concentration was 25 μ g ml⁻¹ and RNA polymerase II concentration was 0.15 μ g ml⁻¹. For RNA-directed RNA synthesis (b) which proved to be two orders of magnitude lower than the DNA-directed RNA synthesis when the same assay conditions were used, the corresponding ³H-labelled NTP was used undiluted. RNA template concentration was 5 μ g ml⁻¹ and RNA polymerase II concentration was 0.75 μ g ml⁻¹. Aliquots (45 μ l) of the reaction mixture were withdrawn at the indicated times and spotted on Whatman 3 MM paper strips (1.5 × 10 cm) which were subsequently chromatographed in saturated (NH₄)₂SO₄/1 M sodium acetate, pH 5.2/isopropanol (50/50/2). The start was cut out and radioactivity was counted in toluene/PPO/POPOP. For each assay five samples were analysed. Circular (C PSTV) RNA and linear (L PSTV) RNA were isolated according to ref. 15.

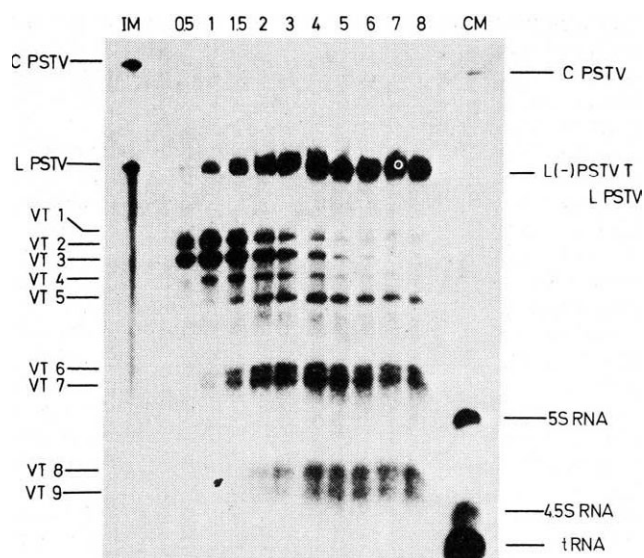


Fig. 2 PAGE analysis of the kinetics of viroid (PSTV)-directed RNA transcription by DNA-dependent RNA polymerase II from tomato. Transcription products after different times of incubation. Conditions for RNA synthesis were as described in Fig. 1 legend. The numbers on top of the slots give the time of incubation in hours. Aliquots (10 μ l) were taken at the indicated time intervals and subjected to PAGE in fully denaturing conditions as described in Fig. 1b legend. Individual bands were visualized by fluorography of dried gels. C PSTV: ¹²⁵I-labelled circular PSTV; L PSTV: ¹²⁵I-labelled linear PSTV; L PSTV T: linear ³²P-labelled PSTV full-length transcript. VT1 migrates close to VT2 and due to overexposure the two bands cannot be discriminated in this autoradiogram.

PSTV RNA molecules were used as templates, the pattern of product formation varied slightly between different template preparations.

Wheat-germ polymerase II seems to transcribe all three viroids practically as efficiently as the tomato enzyme (Table 1), if the overall incorporation is considered, although it is much less efficient in producing full-length viroid (PSTV) RNA copies than is tomato polymerase II (Fig. 1b, lanes 1, 2 and Fig. 3).

Analysis of viroid transcripts

The viroid-directed RNA ³²P-labelled transcription products were analysed on 5% polyacrylamide gels containing 8 M urea¹. When the electrophoresis was carried out in high-salt TEB buffer (89 mM Tris/EDTA/borate, pH 8.3) and at low gel temperature (10 °C), both enzymes gave a very similar RNA product pattern consisting of several distinct bands (Fig. 1a, lanes 1, 2). Surprisingly, transcripts are visible which are apparently larger than the corresponding viroid template itself (Fig. 1a, MTV). However, no reliable conclusions about the actual size of the transcripts can be drawn from this pattern, because the gel provides denaturing conditions only for conventional RNAs, whereas viroid molecules, the corresponding transcripts and their presumptive hybrid complexes with the template remain largely undenatured. If the same products are analysed in low-salt (22 mM) TEB buffer and at high gel temperature (40 °C), where viroid RNA is fully denatured (see ref. 15), circular (C PSTV) and linear (L PSTV) viroid molecules are clearly separated (Fig. 1b, lanes 3-5), and it becomes evident that the largest transcripts from both polymerases have the same migrating properties and hence the same size as the linear viroid molecule. That this RNA is a genuine transcript and not due to terminal labelling of linear viroid molecules was demonstrated by the absence of transcription on omitting one of the labelled triphosphates, and by molecular hybridization.

In addition to the full-length copies about nine smaller distinct viroid transcription products are found of which only transcripts VT1-VT4 are visible in Fig. 1b. Although the tomato and the wheat-germ enzyme synthesize the same size classes of tran-

scripts, densitometric analysis of the autoradiograms shows that their relative amounts differ. After 4 h of RNA synthesis 30% of the total label of the five largest RNA transcripts is found in the tomato enzyme full-length viroid copy, compared with only 9% in the wheat-germ enzyme full-length transcript.

More detailed analysis of the kinetics of tomato polymerase II transcription reveals two classes of RNA products (Fig. 2a): full-length viroid transcripts (L(-)PSTV T) corresponding to the linear viroid molecule (L(+)-PSTV), and viroid transcription products—VT5–VT9 which accumulate with time, and transcripts VT1–VT4 (see also Fig. 1b) which appear very early after initiation of RNA synthesis and decrease subsequently.

Further analysis of the relationship between the different transcriptional intermediates (data not shown) revealed that their assembly into full-length copy occurred within the first 10 min after initiation. Although this rapid synthesis prevents us from determining reliably the actual sequence of the *in vitro* processing by the tomato polymerase, pulse-chase experiments and scanning of the gels clearly demonstrated that the radioactive counts are transferred from species VT1–VT4 to the full-length transcripts. As Fig. 3 shows, in the *in vitro* wheat-germ polymerase system the conversion does not proceed sequentially from the smallest transcript via intermediate-sized molecules to the full-length copies. VT2 and VT3 appear immediately after initiation followed by the formation of VT1 and VT4 (see also Fig. 1b) and the smaller transcripts VT5–VT9 arise at later stages of transcription. These smaller products are also found during transcription with the tomato enzyme (see Fig. 2). Although they are undoubtedly viroid specific, it is still unclear whether or not they are artefacts of the *in vitro* system.

Inhibition and binding experiments

To confirm the highly specific *in vitro* interaction between viroid RNA and polymerase II, inhibition studies with α -amanitin were carried out. This toadstool toxin specifically inhibits polymerase II at a concentration $\sim 10^{-8}$ M *in vitro* and *in vivo*¹⁶; it has no effect on polymerase I¹⁷, but inhibits DNA-dependent RNA polymerase III at 10^{-5} M. RNA-dependent RNA polymerase from healthy tomato tissue, also possibly involved in viroid replication, is insensitive to α -amanitin¹⁸. Figure 4 shows that the transcription of viroid (PSTV) RNA by the tomato polymerase II is inhibited *in vitro* by 10^{-8} M α -amanitin, provi-

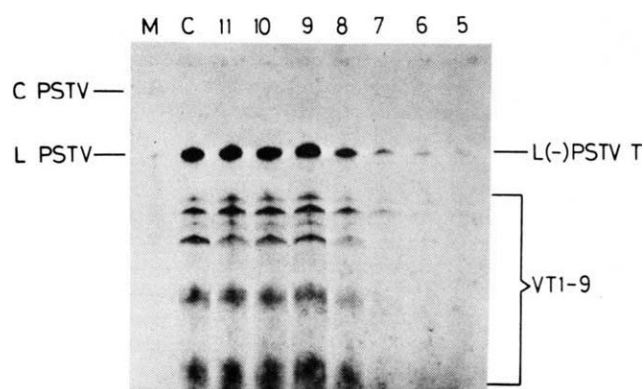


Fig. 4 Inhibition of viroid (PSTV)-directed RNA transcription by α -amanitin as analysed by PAGE. Assay conditions were as described for Fig. 1 in the absence (lane c) and presence of various concentrations of α -amanitin as indicated on top of the lanes, where the numbers correspond to the negative log₁₀ of the molar concentrations of α -amanitin present in the assay. The assay mixture was incubated for 2 h and 10- μ l aliquots were analysed on the gel as described in Fig. 1b legend. C, Control without α -amanitin; M, circular and linear ¹²⁵I-labelled PSTV marker RNA; VT1–VT9, viroid (PSTV) RNA-directed RNA transcripts.

ding clear evidence that the *in vitro* transcription of the viroid molecule proceeds with the aid of DNA-directed RNA polymerase II and is not based on the contaminating activity of any other known nucleic acid-synthesizing enzyme.

We investigated the complex formation between ¹²⁵I-labelled viroid (PSTV) RNA and tomato polymerase II using the nitrocellulose filter binding technique¹⁹, in which the complex of nucleic acid template and polymerase is retained on the nitrocellulose whereas uncomplexed template is washed through the filter.

Figure 5a shows that a binary complex between the viroid template and the polymerase II is formed and that binding is a function of the polymerase concentration. Both circular and linear viroid (PSTV) molecules show identical binding properties. Furthermore, the binding of ¹²⁵I-labelled PSTV RNA to the polymerase decreased with increasing concentrations of poly d(A-T) (Fig. 5b), suggesting that viroid RNA binds to the same template-binding site on the polymerase II molecule as do genuine DNA templates. This was tested with poly d(A-T) as template in the presence of viroid RNA; in this case only UTP and ATP are required for RNA synthesis and so viroid RNA-directed RNA transcription requiring all four nucleoside triphosphates will be suppressed in this assay. With increasing viroid RNA concentrations, poly r(A-U) synthesis is indeed drastically reduced (Fig. 5c). All these results demonstrate a specific interaction between viroid RNA and polymerase II.

Molecular hybridization

Because of the high self-complementarity of the viroid molecule, experimental conditions were used which prevented self-annealing during hybridization. Thus, RNAs were separated on 5% polyacrylamide gels in fully denaturing conditions (see Fig. 1b legend) and after alkali-treatment were blotted and covalently linked to diazobenzoyloxymethyl (DBM)-paper according to Alwine *et al.*²⁰. Cellular RNAs from both healthy and PSTV-infected plants were fractionated into 2 M LiCl-soluble and -insoluble (ribosomal) RNAs and subsequently treated with DNase I to remove any DNA contamination. Radioactively labelled viroid and cellular RNAs were run as markers on the same gel.

Two approaches were taken: (1) blotting of unlabelled viroid and cellular RNAs followed by hybridization with ³²P-labelled PSTV transcripts, and (2) blotting of viroid, cellular and transcribed RNAs and hybridization with ¹²⁵I-labelled PSTV (Fig. 6a and b, respectively). From both analyses we conclude: First,

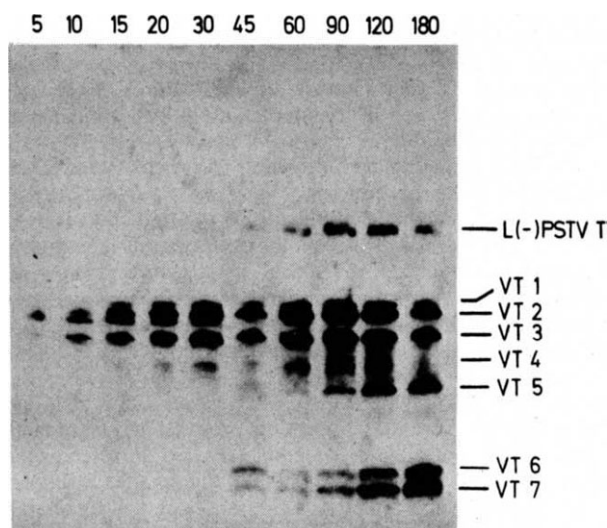


Fig. 3 Analysis of the kinetics of viroid (PSTV)-directed RNA transcription by DNA-dependent RNA polymerase II from wheat germ. Experimental conditions were as described in Figs 1 and 2 legends. The numbers on top of the slots give the time of incubation in min. The kinetics of transcription with the wheat-germ polymerase resemble a 'slow motion picture' of the more rapid processing by tomato polymerase where all transcriptional intermediates are found within 10 min after initiation. L (-) PSTV T: linear full-length viroid (PSTV) transcript, VT1–VT7: viroid (PSTV) RNA-directed RNA intermediates.

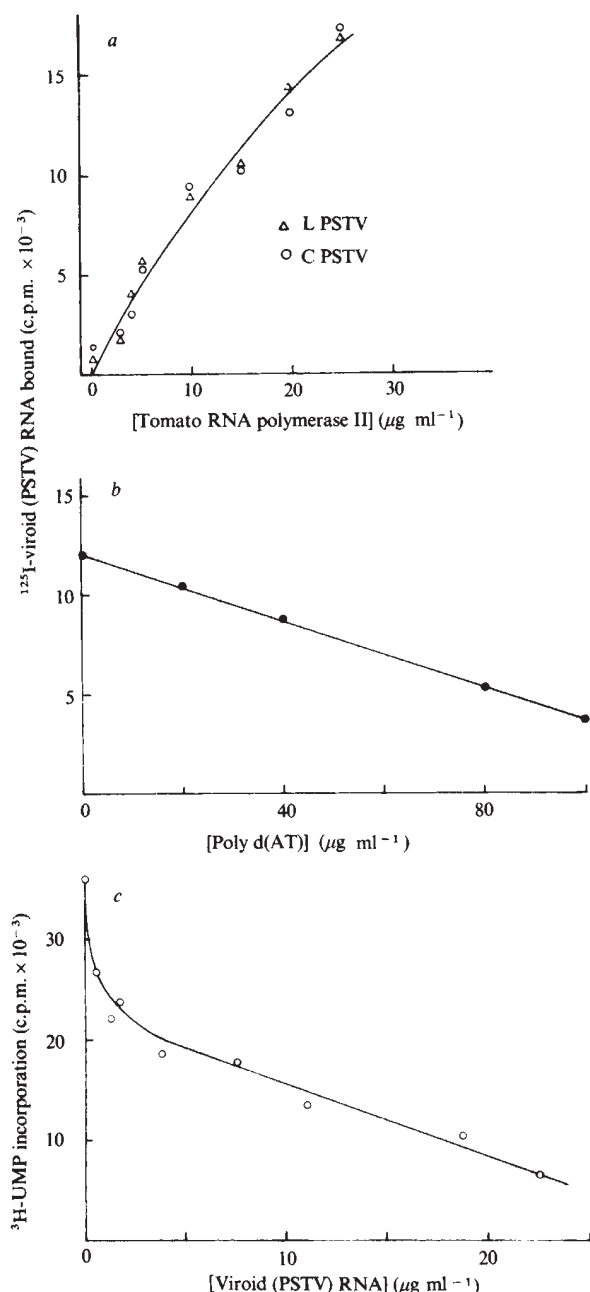


Fig. 5 Analysis of binding between DNA-dependent RNA polymerase II from tomato and viroid (PSTV) RNA. Nitrocellulose filter assay to demonstrate the binary complex formation between tomato DNA-dependent RNA polymerase II and viroid (PSTV) RNA by binding experiments (a) and by competition experiments (b) with poly d(A-T). The binding experiments were performed as described by Seidman *et al.*¹⁹, but the polymerase assay buffer (see Table 1 legend) containing 5% dimethyl sulfoxide DMSO was used as binding buffer. The total amount of ^{125}I -labelled viroid (PSTV) RNA was 35,000 c.p.m. (specific activity 3.5×10^7 c.p.m. per μg) per individual assay. For the competition experiment the concentration of viroid (35,000 c.p.m.) and of tomato polymerase II ($15 \mu\text{g ml}^{-1}$) was kept constant whereas the poly d(A-T) template was added to the assay mixture as indicated in b. c, Inhibition of tomato DNA-dependent RNA polymerase II transcription by viroid (PSTV) RNA. The assay was performed as described in Table 1 legend. The only NTPs present in the reaction mixture were ATP (400 μM) and ^3H -UTP (40 μM). Polymerase concentration was $1.5 \mu\text{g ml}^{-1}$. The assay mixture containing viroid (PSTV) RNA as indicated in c and all compounds necessary for RNA synthesis except DNA template was pre-incubated 10 min at 37°C . The transcription was then started by the addition of poly d(A-T) template ($25 \mu\text{g ml}^{-1}$) and the assay mix was incubated for 20 min at 37°C .

transcription of PSTV by DNA-directed RNA polymerase II yields genuine RNA transcripts which hybridize to unlabelled linear and circular PSTV (Fig. 6a, lane 4). This is also evident from the reciprocal hybridization of ^{125}I -PSTV to covalently

fixed, unlabelled PSTV transcripts (Fig. 6b, lanes 4, 6). Only a few of the viroid precursor RNAs are visible (Fig. 6b, lanes 4, 6), because their concentration is low relative to that of the viroid template still present in the reaction mixture. Furthermore, most of the transcripts are full-length RNAs because of the increase of reaction time and ribonucleoside triphosphate concentration. Second, unlabelled linear and circular PSTV hybridize not only with their ^{32}P -labelled complementary (-) RNA transcripts (Fig. 6a, lane 4), but also intermolecularly with ^{125}I -labelled PSTV exhibiting the same (+) polarity (Fig. 6b, lane 5), thus clearly demonstrating that molecular hybridization in standard conditions cannot distinguish between (+) and (-) viroid sequences, and therefore previously published data on this question^{21,22} must be interpreted with caution. Third, whereas healthy tomato plants (Fig. 6a, lanes 2, 3 and 6b, lanes 7, 8) do not show any detectable viroid (+) or (-) sequences in the 2 M LiCl-soluble and -insoluble fractions, PSTV-infected tomato plants contain RNA species in both soluble (Fig. 6a, lane 5; Fig. 6b, lane 2) and insoluble (Fig. 6a, lane 6; Fig. 6b, lane 3) fractions that hybridize with (+) and (-) viroid RNA. These presumptive replicative intermediates occurring *in vivo* are large and do not enter the 5% gel, but can be resolved on 3.5% agarose gels into at least seven species larger than viroid RNA (see ref. 30). The exact precursor relationship, however, between these larger, viroid-specific RNA species and linear and/or circular PSTV remains to be established.

The accumulation of predominantly circular viroid molecules in plant tissue^{1,15} indicates that the process of viroid replication via linear replicative (-) and (+) intermediates must involve a final step of circularization. Irrespective of this still unknown *in vivo* ligation, $5'$ - ^{32}P -labelled linear PSTV can be ligated *in vitro* by the conventional bacteriophage T4-induced RNA ligase to form circular PSTV molecules (Fig. 6c).

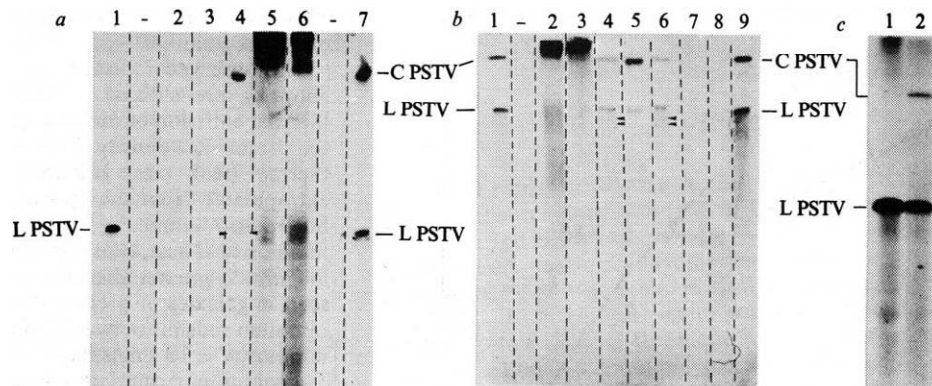
Discussion

From the data in this and our previous papers we conclude that the first step in the replication process—the copying of the infecting viroid RNA molecule—proceeds with the aid of the DNA-dependent RNA polymerase II of the host plant. The finding that a DNA-dependent polymerase can quite efficiently accept and transcribe viroid (+) RNA into its complementary (-) RNA form may seem surprising. However, the abilities of viroid RNA to form a binary complex with the polymerase, to compete with DNA for the template binding site on the enzyme, and to inhibit DNA-directed RNA synthesis strongly suggest that the previously described DNA-like features of the viroid molecule are involved in its interaction with the polymerase. Therefore, it is intelligible from a mechanistic point of view that viroid RNA is capable of directing DNA-dependent RNA polymerase for its replication.

From the appearance of the distinct viroid-specific RNA transcripts VT1-VT4 during synthesis of the full-length viroid copy we conclude: first, the secondary structure of the viroid molecule causes the enzymes to pause at specific sites analogous to the transcription pattern of Q β midvariant MDV-1 RNA by Q β replicase²³, and second, specific recognition sites must exist in the viroid RNA molecule for both RNA polymerases. It is unclear why the polymerase stops after one round of *in vitro* transcription, whereas transcription products larger than the viroid molecule itself are detectable *in vivo*. Further experiments are needed to decide whether the smaller transcripts VT5-VT9 which accumulate later in transcription are caused by a site-specific nuclease activity possibly present in the polymerase preparation or whether they are transcripts of specifically nicked viroid molecules, in which case the transcription would stop at the nick.

The demonstration that viroids replicate and accumulate in the nucleus^{8,24-26}, and most efficiently in rapidly dividing plant tissue²⁷, may now be related to the fact that, due to its normal function of synthesizing genomic messenger RNAs, polymerase II is highly active in the nuclei of dividing cells, and it would

Fig. 6 Molecular hybridization of radioactively labelled (+) and (-) PSTV RNAs to RNAs covalently bound to DBM-paper. Preparation of DBM-paper, blotting in 25 mM sodium phosphate buffer, pH 6.8, and hybridization followed the procedure of Alwine *et al.*¹⁷. Separation of RNAs on 5% polyacrylamide gels in fully denaturing conditions was achieved as described in Fig. 1b legend. 4 µg of unlabelled PSTV RNA and 500 µg of each RNA fraction were applied; linear and mixtures of linear and circular ¹²⁵I-labelled PSTV served as markers and were also blotted onto the paper. Hybridization was performed at 41.5 °C for 48 h with 5 × 10⁴ c.p.m. cm⁻² paper surface area of ¹²⁵I-labelled PSTV and with 3 × 10⁵ c.p.m. cm⁻² paper surface area of ³²P-labelled PSTV transcripts. **a**, Hybridization with ³²P-labelled PSTV transcripts. Lanes 1, 7: ¹²⁵I-PSTV marker; 2, LiCl-soluble RNA from uninfected tomato; 3, rRNA from uninfected tomato; 4, unlabelled viroid (PSTV) RNA; 5, LiCl-soluble RNA from PSTV-infected tomato; 6, rRNA from PSTV-infected tomato. **b**, Hybridization with ¹²⁵I-labelled PSTV RNA. Lanes 1, 9, ¹²⁵I-PSTV marker; 2, LiCl-soluble RNA from PSTV-infected tomato; 3, rRNA from PSTV-infected tomato; 4, 6, unlabelled PSTV RNA transcripts; 5, unlabelled PSTV RNA; 7, LiCl-soluble RNA from uninfected tomato; 8, rRNA from uninfected tomato. **c**, Circularization of ³²P-labelled linear PSTV by T4-induced RNA ligase. PSTV RNA (2 µg) containing 1–2% of linear PSTV was dephosphorylated by acid phosphatase from human prostate and labelled at its 5'-position by the action of polynucleotide kinase in the presence of [γ-³²P]ATP (specific activity 1,000 Ci mmol⁻¹). The ligase reaction was carried out in a total volume of 25 µl containing 50 mM Tris-HCl pH 7.9, 20 mM MgCl₂, 0.1 mM ATP, 3.3 mM dithiothreitol, and 10 units of T4-induced RNA ligase. After 30 min at 37 °C, the reaction was stopped by the addition of EDTA to 50 mM, and the reaction products were analysed as described in Fig. 1b legend. Before autoradiography, gels were stained with methylene blue to visualize unlabelled circular PSTV. Lane 1, 5-³²P-labelled linear PSTV RNA before ligation; lane 2, ³²P-labelled PSTV RNA after ligation.



hence be available there for intensive viroid replication. Viroid pathogenesis could also be explained in these terms. As the infecting viroid molecule directs the nuclear DNA-dependent RNA polymerase II to perform its 'selfish' replication the synthesis of genomic messenger RNAs of the host cell may become inhibited or repressed. Thus, normal differentiation would be disturbed, leading to the expression of the typical symptoms of leaf malformation and retardation of growth.

We thank Dr H.-P. Mühlbach for the continuous supply of tomato callus tissue and for helpful advice, Drs F. Boege, and D. Riesner for critical discussions, Mrs M. Bahr, M. Jung, H. Muth, K. Ramm and Mr H. Thiele for technical assistance and Mrs C. Reitz for photographic and drawing work. Our investigation was supported by the Deutsche Forschungsgemeinschaft through the Sonderforschungsbereich 47, and through a Heisenberg Fellowship given to W.R.

Received 30 September 1980; accepted 25 February 1981.

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LETTERS TO NATURE

Millimetre observations of quasar Q0420–388

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The quasar, Q0420–388, ($Z = 3.12$, $m = 16.9$, ref. 1) was detected at a wavelength of 1 mm during part of a programme of 1-mm observations of optically selected ($m < 17.5$) quasars². We observed a strongly inverted spectrum and confirm the high luminosity³. The observations support the explanation of the radio-quiet quasar phenomenon as being due to very compact structures which are self-absorbed even at short centimetre wavelengths.

The observations were made at the prime focus of the European Southern Observatory (ESO) 3.6-m telescope on La Silla at two epochs: 9–11 September 1979 and 1–7 July 1980. The detector for both epochs was a composite bolometer (Infrared Laboratories) with a measured noise equivalent power (NEP) of $9.6 \pm 1.0 \times 10^{-5}$ W Hz^{-1/2}. It was cooled to 1.2 K with superfluid helium. The sensitivity, signal-to-noise ratio of 1 in 1 h was 0.7 Jy in 1979 and 0.5 Jy in 1980. The improvement was a result of changing the filter from two capacitive meshes with a maximum transmission of 45% and half-power cuton at 650 µm to one with four capacitive meshes⁴ with 75% transmission and cuton at 710 µm as measured with a Michelson interferometer. The horn exit aperture determined⁵ the long wavelength cutoff at 2.1 mm.

To reduce the influence of background drift the data were taken in cycles, ABBA, where first beam A was directed on the source for 10 s then, after a 3-s delay to ensure that any electrical

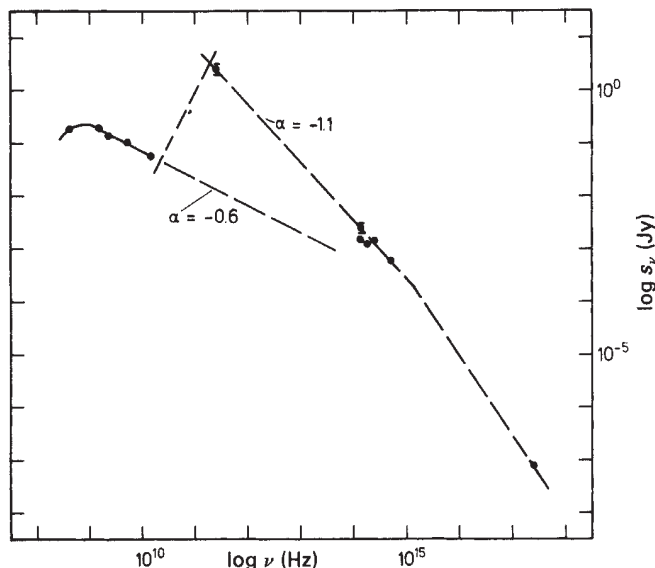


Fig. 1 The spectrum of Q0420-388. The radio data are from refs 15, 16 and have errors $\leq 10\%$. The IR point with the 20% error bar is from ref. 3 while the other IR data are from ref. 8 with errors $\sim 10\%$. The optical¹ and X-ray¹³ data also have errors of $\sim 10\%$. The millimetre flux density for September 1979 is shown with an error bar of $\pm 30\%$. The error in the frequency is discussed in the text.

interference had decayed, beam B was directed on source for a pair of 10-s integrations. The phase was such that, for a signal, $A-B$ was equal to twice the true signal. At both epochs there was an excess number of positive pairs, $A-B > 0$, above that expected if the sign of the measurements was randomly distributed: in 1979, from 324 pairs, 186 were positive and in 1980, from 612 pairs, 401 pairs were positive. There is, therefore, no doubt that the source has been detected. The signal was chopped by means of a sector mirror mounted on an air bearing and rotating at 15 Hz (ref. 6). The measured beam size was 2 arc min (FWHP) and the separation of the beams was 2.25 arc min.

The extinction and calibration are determined from observations of the planets. The effective frequency is determined both by the spectral index of the source and by the precipitable water vapour content of the atmosphere as determined from the extinction⁷. The latter indicated 3 mm of water for the September data and the optical/IR data^{1,3,8} suggested a spectral index of -1.1 ; the effective frequency is 245 GHz (1.2 mm). An increase of 2 mm in the water vapour content would decrease the frequency by only 6%. Increasing the spectral index to $\alpha = 0$ increases the frequency to 268 GHz and leaves the flux density unchanged. Thereafter, increasing the spectral index by 1 increases the frequency by $\sim 8\%$ and the flux density by $\sim 15\%$.

The measured 1-mm flux densities for Q0420-388 were:

9-11 September 1979	2.5 ± 0.7 Jy
1-2 July 1980	6.3 ± 0.8 (Preliminary)
3 July	3.6 ± 1.0 (Preliminary)
5 July	6.7 ± 0.9 (Preliminary)
7 July	5.2 ± 0.7 (Preliminary)

The mean values for 9-11 September 1979 and 1-7 July 1980 are significantly different and indicate that real variations have occurred over ~ 300 days. The September value is shown in Fig. 1.

The IR data also contain the suggestion of variability (Fig. 1). A comparison of the K magnitudes found by Wright and Kleinmann³ (13.5 ± 0.2) and by Glass⁸ (14.07 ± 0.09) show that at the 98% confidence level the magnitudes are different. Wright and Kleinmann³ showed that during the past 40 years the optical variations have not differed from the mean by more than a factor of 2. However, photometric coverage was inadequate to detect short-term changes and the time scale for optical and IR variability remains to be determined. Our millimetre data are

consistent with the size of the variations seen in the IR and optical region.

The observed radiation has a rest wavelength of $\sim 300 \mu\text{m}$ adopting a redshift of $z = 3.12$. If this radiation were from the thermal emission of dust it would have to be a coincidence that the 1 mm flux density from the long wavelength side of the thermal peak (at $\leq 100 \mu\text{m}$) agrees so well with the value extrapolated from the optical/IR region. Not only would the bolometric luminosity be considerably increased but also the luminosity of dust, in excess of $10^{15} L_{\odot}$, would be many orders of magnitude greater than the upper limits given for quasars or seen in galaxies or predicted for primeval galaxies even for an optimum redshift between 5 and 10 (refs 9-12). The millimetre luminosity is 10 times larger than that of the X rays¹³ (Fig. 2). The addition of our data suggest that the total luminosity ought to be increased by at least a factor of 4 over that previously reported³ to $3 \times 10^{15} L_{\odot}$ assuming $q_0 = 0$, and $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$. ($F_{\text{bol}} = (4.16 \pm 0.04) \times 10^{-14} \text{ W m}^{-2}$ integrating from $\sim 24 \text{ GHz}$ to $4.8 \times 10^{17} \text{ Hz}$). Using the July data would increase these values a further 70%.)

Q0420-388 is not listed in the Parkes catalogue¹⁴ but it has recently been detected between 408 MHz and 14.5 GHz (refs 15, 16), the data being obtained quasi-simultaneously over a few months in 1978. With the radio and millimetre data the quasar has an inverted spectrum between 14.5 and 245 GHz ($\alpha \geq +1.3$) (Fig. 1) and probably below $\sim 408 \text{ MHz}$. This spectrum may be interpreted as originating from synchrotron self-absorbed components with turn-over frequencies near 560 MHz and 140 GHz. Although VLBI observations are required to measure the angular diameters of the components, the diameters may be estimated from the spectrum if some assumptions are made. Where VLBI data of compact components exist the magnetic fields are typically $10^{-4.1} \text{ G}$ (ref. 17). However, in the case of Q0420-388 the magnetic energy would be insufficient to cause the synchrotron radiation. Therefore to estimate the physical size we adopt the condition that the energy in relativistic particles, E_p , be comparable with that in the magnetic field, E_m . In Table 1, the results are given, beginning with the trial value of 10^{-4} G . The magnetic field in the large component must be stronger than 10^{-3} G to achieve equipartition.

This field was used as a trial value for the small component. However, the energy in relativistic particles would then be more than 10^{11} times that in the magnetic field. A field in excess of 0.1 G is required for equality. The trend for the smallest sources to have the highest magnetic fields has been noted previously¹⁷. It may also be noted in Table 1 that, in this condition of approximately equal energy distributions, only 1% of the energy is in the small component (10^{55} erg compared with 10^{57} erg). Also the radiating lifetime is uncomfortably short: $\sim 3 \text{ yr}$, implying a scale size $3 \times 10^{18} \text{ cm}$ for electrons travelling at the speed of light.

The derived linear sizes are given in Table 1 for $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and $q_0 = 0$. For a magnetic field of 10^{-1} G the

Table 1 Physical properties of Q0420-388

Large component ($S_{\nu} = 0.33 \text{ Jy}$ at turnover $\nu = 560 \text{ MHz}$)				
B (G)	θ (m arc s)	E_p (erg)	E_m (erg)	E_p/E_m
10^{-4}	~ 2.5 ($1.0 \times 10^{20} \text{ cm}$)	$\sim 9 \times 10^{59}$	$\sim 4 \times 10^{53}$	$\sim 2 \times 10^6$
10^{-3}	~ 4.4 ($2 \times 10^{20} \text{ cm}$)	$\sim 5.5 \times 10^{57}$	$\sim 2 \times 10^{56}$	~ 20
Small component ($S = 4.3 \text{ Jy}$ at turnover $\nu = 140 \text{ GHz}$)				
10^{-3}	$\sim 1.6 \times 10^{-2}$ ($7 \times 10^{17} \text{ cm}$)	$\sim 4 \times 10^{60}$	$\sim 1 \times 10^{49}$	$\sim 4 \times 10^{11}$
10^{-1}	$\sim 5.0 \times 10^{-2}$ ($2 \times 10^{18} \text{ cm}$)	$\sim 1 \times 10^{56}$	$\sim 3 \times 10^{54}$	~ 40
(July maximum; $S_{\nu} = 5.8 \text{ Jy}$ at turnover $\nu = 300 \text{ GHz}$)				
0.5	$\sim 3.4 \times 10^{-2}$ ($1 \times 10^{18} \text{ cm}$)	$\sim 8 \times 10^{54}$	$\sim 3 \times 10^{55}$	~ 0.3

Linear size based on $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and $q_0 = 0$.

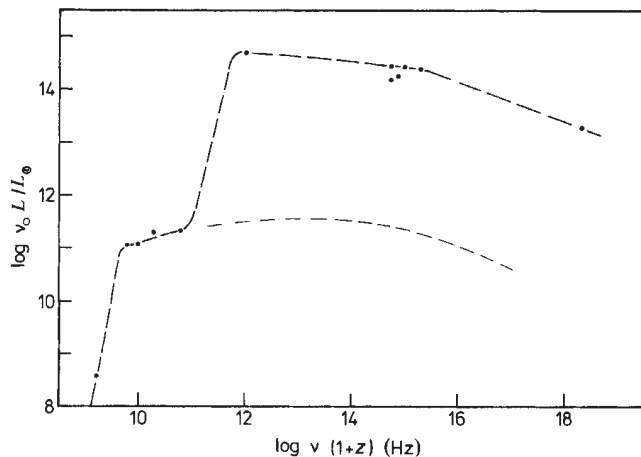


Fig. 2 Luminosity of Q0420–388. The luminosity is given by $\nu S_{\nu} 4\pi(c/H_0)^2 z^2 (1+z/2)^2 (1+z)^{-(1+\alpha)} / L_{\odot}$ for $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and $q_0 = 0$, $z = 3.12$. The spectral indices were taken from Fig. 1. Note that $\alpha = 2.5$ was assumed for the 408 MHz point and $\alpha = -1.5$ for the X-ray point at $4.8 \times 10^{17} \text{ Hz}$.

millimetre source has a size of 10^{18} cm in agreement with its radiating lifetime and the scale of millimetre variability. It is obvious that this point needs further study and additional confirmation.

We thank Drs Michael Arnold and Hans Peter Gemünd for work on improving filters for 1 mm, also Drs Dave Jauncey and Ian Glass for their radio and IR data and Drs Martin Harwit, Ivan Pauliny-Toth, Peter Strittmatter and Arno Witzel for discussions. This work is supported by the Deutsche Forschungsgemeinschaft, SFB 131.

Received 3 November 1980; accepted 25 March 1981.

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A period–luminosity relation for Mira variables in the Large Magellanic Cloud

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Mira variables are very bright objects in the IR ($\sim 2\mu\text{m}$) so that they can be seen to large distances and through heavy interstellar absorption. Provided that they have well defined luminosities they should therefore be ideal indicators of galactic and extragalactic distances. Luminosities are also essential for understanding the physical state and pulsational characteristics of these stars which may be at the final stage of stellar evolution before planetary nebula formation^{1–3}. A period–luminosity relation has been established for galactic Miras⁴ but the intrinsic scatter in the relation is largely unknown. We report here periods and bolometric magnitudes for Miras in the Large Magellanic Cloud (LMC) which show a period–luminosity relation with very small scatter.

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A search for red variables on visible light photographs of the Magellanic Clouds has been described elsewhere⁵; 12 Mira variables were found in a field of 0.3 deg^2 in the Bar of the LMC and periods with an error of ~ 10 days were determined. Details will be published elsewhere. Each star was observed at least three times in the period 1976–80 using the IR photometer⁶ on the 1.9-m reflector at Sutherland. An InSb detector was used and the system is believed to be linear to better than 0.1 mag. The mean standard errors of single observations at J , H and K are 0.07, 0.04 and 0.04 mag respectively. Confusion by stars in the reference beam prevented satisfactory results for one star, which has been omitted from the discussion, and may also be responsible for the excessive red colour found for number 153. The observations do not define the light curve as a rule, so that the mean J , H and K magnitudes were found and the bolometric magnitude estimated by fitting a black-body curve⁴. The results are given in Table 1. The mean range of K in the present data is 0.5 mag, so the error in a bolometric magnitude will exceed the purely photometric errors noted above.

The 11 stars are assumed to be a homogeneous sample of M type Miras similar to those in our own Galaxy. The colours in the visible and IR regions are consistent with this except for C7 which has been found spectroscopically to be a carbon star (T. L. E., in preparation). Its exclusion would make virtually no difference to the adopted period–luminosity relation.

The data of Table 1 are plotted in Fig. 1, which also shows the best fitting straight line. This has the equation

$$m_{\text{bol}} = 19.25 - 2.09 \log P \text{ (days)}$$

The r.m.s. deviation of a single star is only 0.22 mag, much of which is attributed to the small number of observations. A selection effect cannot be ruled out for the longer period stars, which are faint visually, but the sample is believed to be complete for those with $P \leq 300$ days. The intrinsic scatter may thus be very small and is less than the value of 0.25–0.30 mag found for classical Cepheids if no colour term is included⁷. This in turn implies that the instability strip is narrow.

The LMC distance modulus from classical Cepheids is 18.69 mag (ref. 7), so the absolute bolometric magnitudes are

$$M_{\text{bol}} = 0.56 - 2.09 \log P$$

The slope of the period–luminosity relation may be fitted to the bolometric magnitude calibration for galactic Miras⁴ to give

$$M_{\text{bol}} = 0.76 (\pm 0.11) - 2.09 \log P$$

Double weight has been given to the 12 stars in ref. 4 with individually known distances. The difference between the zero points of the galactic and Magellanic calibrations is not significant. Adoption of the galactic zero point would imply a distance modulus for the LMC of 18.5 mag.

The question arises whether the application of this result to distance determination would be vitiated by intrinsic differences between the stars observed in different systems. Feast⁸ has argued that there is little possibility of confusion between Mira

Table 1 Bolometric magnitudes of Mira variables

Star	P (days)	m_{bol}
C38	131	14.85
C48	175	14.18
C11	200	14.40
C20	207	14.57
120	213	14.40
141	260	14.13
110	266	14.60
C7	314	14.03
153	360:	14.13:
49	390	13.67
105	430	13.51

The star names are running numbers and the numbering system has the same significance as that in ref. 5.

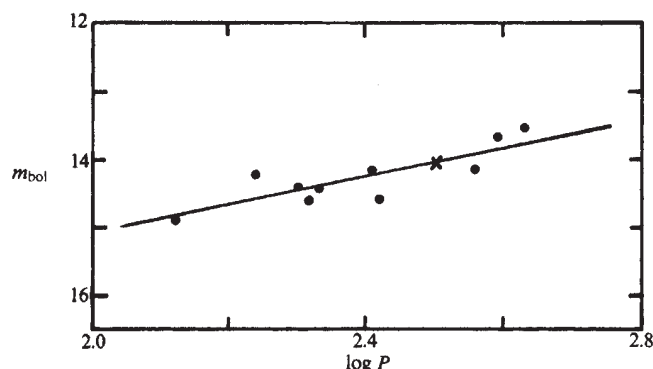


Fig. 1 m_{bol} for LMC Miras plotted against $\log P$ (days). $\times$, The carbon star. The best fitting linear regression line is shown.

variables and the red supergiant variables of large amplitude. There is a possibility, as in the case of other objects used as standard candles, that the luminosities depend on metal abundance. The satisfactory agreement between the zero points found for Mira variables in the Galaxy and the LMC indicates that any such difference is small.

Observations of a larger sample of stars, with more complete coverage of the light cycle, should allow an improved calibration to be found from Magellanic Cloud data alone. It seems clear from our results that Mira variables should indeed be good distance indicators.

We thank Dr M W Feast for valuable suggestions.

Received 12 January; accepted 19 March 1981.

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Modulation of the Earth's electric field by cosmic radiation

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Positive correlations between cosmic radiation and ionospheric potential (V_i) reported here indicate that the electrification of the atmosphere is modulated by changes in ionizing radiation. Analyses of two independent V_i data sets, where V_i is a measure of the intensity of the Earth's electric field^{1,2}, indicate that a 10% change in ground level cosmic radiation is associated with a 10–20% corresponding variation in V_i . In addition, variations in V_i were analysed relative to indices of the upper atmosphere electrical generator to investigate possible atmospheric electrical changes due to magnetospheric–ionospheric coupling; no correlation was found between the upper atmosphere indices and V_i . The nature of the global circuit is such that ionizing radiation must affect atmospheric electrification through regulation of the current output of the global thunderstorm generator which maintains the fair-weather electric field because increased conductivity in the fair-weather part of the atmosphere would lower V_i . The results also suggest the existence of a thundercloud electrification mechanism that is sensitive to ambient atmospheric electrical conditions—electric field intensity and/or conductivity^{3,4}.

An atmospheric electrical mechanism is a promising approach to the Sun–weather problem because it offers the possibility for solar control of energetics in the lower atmosphere without requiring energy transfer through the upper atmosphere or a change in the solar constant^{5,6}. The energy distribution in the troposphere must be modified if weather is to be affected. If the Earth's electric field and/or ionization in the lower stratosphere were altered, this could influence cloud physical processes releasing latent heat and thus affecting atmospheric dynamics^{7,8}. Another important characteristic of an electrical mechanism is that a rapid (<1 day) atmospheric response would be expected. Thus, it could explain meteorological effects that have been reported to follow within 1 day of solar flares^{9–12} and magnetic sector crossings^{13,14} which cannot be explained by heating mechanisms. The rapid response of an atmospheric electrical Sun–weather mechanism enables such a mechanism to be tested critically, whereas Sun–weather mechanisms depending on heating and planetary waves¹⁵ have long (weeks to years) undefined time-delays and would be difficult to test experimentally.

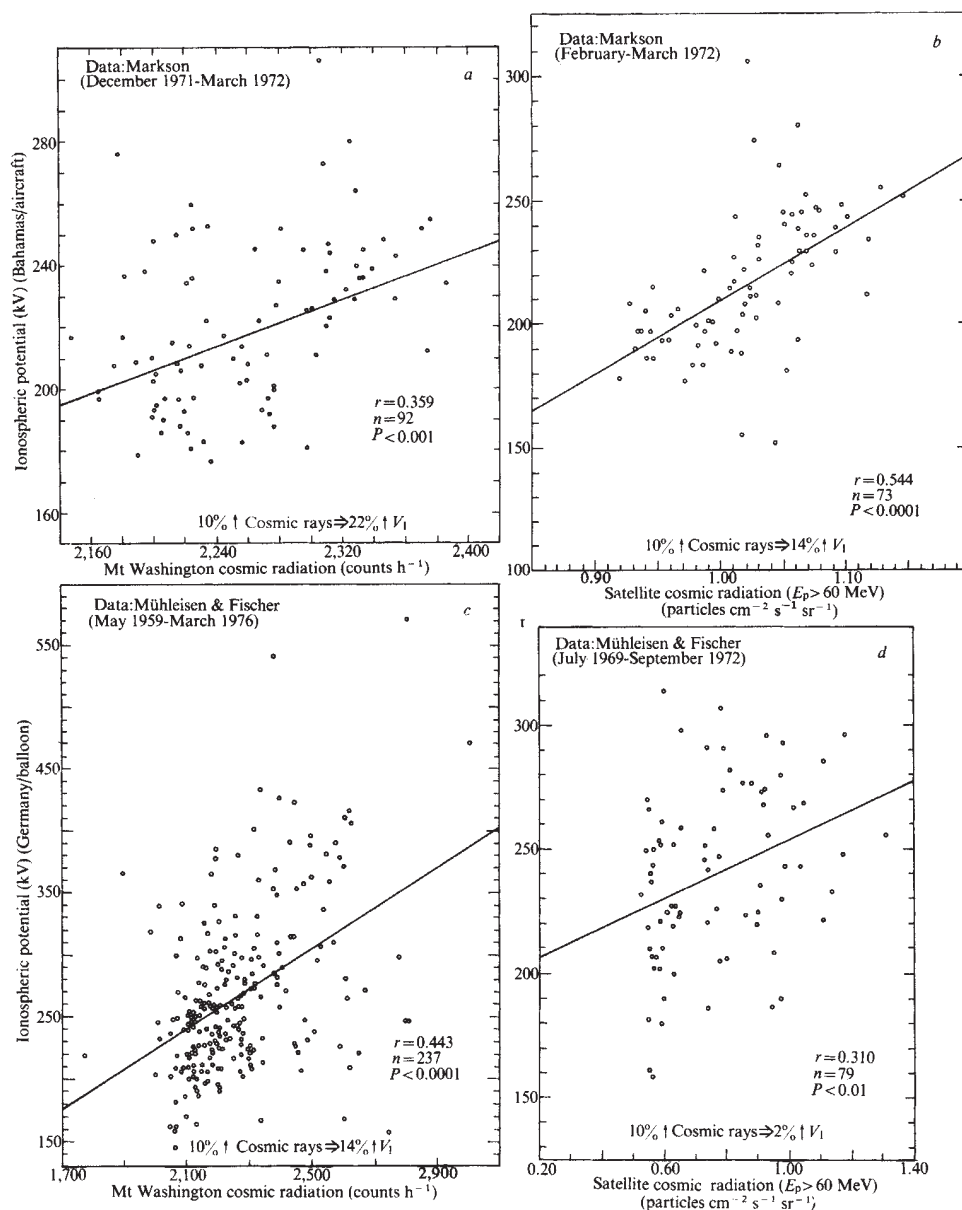
Recently, solar wind velocity has been introduced as a parameter in Sun–weather research to examine the relationship between the fine structure of the interplanetary medium and atmospheric parameters¹⁶. The inverse correlation found between solar wind velocity and both cosmic radiation and V_i was taken to infer that cosmic radiation and V_i were positively correlated and that the former controlled the latter. It was postulated that discontinuities in the interplanetary magnetic field (frozen in the solar wind), which modulate cosmic radiation, cause changes in stratospheric conductivity and thus regulate currents flowing from the global thunderstorm generator to the ionosphere. These currents maintain V_i and the Earth's electric field. To investigate the assumed cosmic radiation– V_i relationship, these parameters have now been directly compared and the predicted positive correlation has been found.

Two disparate sources of cosmic radiation were correlated with ionospheric potential. The first was from the Mount Washington, New Hampshire, neutron monitor located at a height of 800 mbar (these data cover the period 1954 to the present). The median in the response curve of this instrument is 6 GeV and it is capable of sensing particles down to the geomagnetic cutoff of 0.7 GeV. The second set of cosmic ray data came from the IMP 4 satellite and constitute the longest available coherent set of satellite data including the period of the Bahamas V_i soundings used previously in the solar wind study¹⁶. The period June 1969–December 1972 (all the available data) was analysed. Hourly count rates were available in each data set. With the satellite data, only count rates for the most energetic particles ($E_p > 60$ MeV) were considered. Energetic particles trapped in the Earth's radiation belts and a few instances of solar protons had been removed from these data so that the record represents only the rate of galactic cosmic radiation.

The V_i data came from two independent sources. The first was obtained in the Bahamas using an aircraft during the period December 1971–March 1972¹⁷. Of the 94 total soundings made in the Bahamas region, 92 occurred when the Mount Washington neutron monitor data were available and 73 when the satellite was measuring cosmic radiation. Only data taken in the Bahamas and ocean regions off the coast of Florida were used in the analysis when it was found that meteorological factors inherent to conditions over land (mostly related to convection and pollution) introduced error into the 26 soundings made over the north-eastern United States, Nova Scotia and adjacent ocean areas. These factors will be discussed elsewhere.

The second V_i data set, provided by Mühleisen and Fischer¹⁸, consists of a list of 246 V_i balloon soundings obtained mostly in Germany between 1959 and 1976. For these measurements there were 237 corresponding observations of cosmic radiation rates from Mount Washington and 79 from the satellite. The Bahamas and German V_i soundings are the only existing data sets of ionospheric potential variation that are sufficiently extensive and coherent to be useful in solar–terrestrial cor-

Fig. 1 Scatter diagrams of ionospheric potential (V_I) compared with cosmic radiation rates. *a*, Bahamian V_I data versus Mount Washington cosmic radiation. *b*, Bahamian V_I data versus satellite cosmic radiation. *c*, German V_I data versus Mount Washington cosmic radiation. *d*, German V_I data versus satellite cosmic radiation.



relation studies. It has been suggested that this parameter should be measured routinely or continuously to provide a "geoelectric index" to complement the "geomagnetic index"¹⁶.

Figure 1*a* is a scatter diagram of the Bahamas ionospheric potentials compared with simultaneous Mount Washington neutron counts. The cosmic ray rates are interpolated to the times of the V_I soundings from the previous and next hour values. The correlation coefficient is 0.359 which for 92 independent data points would be significant at the 0.001 level. However, these data are not fully independent and it is difficult to estimate accurately the proper degrees of freedom for statistical testing. The effects of persistence in data on probability estimates will be considered elsewhere. Each part of Fig. 1 depicts the per cent change in the dependent variable (V_I) based on a 10% increase in the independent variable (cosmic radiation). This is determined from the slope of the least squares line of regression. In this case, a 10% increase in the Mount Washington neutron monitor rate is associated with a 22% increase in V_I .

Figure 1*b* is the complementary analysis using the satellite cosmic ray data. Here the correlation of 0.544 is larger than for the cosmic radiation measured on the ground and a 10% increase in cosmic radiation corresponds to a 14% increase in V_I .

For the balloon soundings of Mühleisen and Fischer¹⁸, Fig. 1*c* indicates a 0.443 correlation with a 10% increase in Mount Washington cosmic radiation corresponding to a 14% increase in V_I . No interpolation was necessary with these data as their times are listed as on the hour. Figure 1*d* shows the correlation between these V_I soundings and the satellite data is 0.310 with a 10% increase in cosmic radiation associated with a 2% increase in V_I .

Figure 2*a* shows that the cosmic ray modulation was not present in the soundings made during the aircraft measuring programme in places other than the Bahamas and Florida coastal waters. These data illustrate the importance of site location in obtaining V_I measurements that are representative of global conditions.

It is of interest to compare the satellite data with Mount Washington count rates. Figure 2*b* shows that this correlation is ~ 0.9 with the satellite instrument twice as sensitive as the neutron monitor to changes in galactic cosmic radiation. This ratio should vary for data obtained at other phases of the sunspot cycle because of changes in the cosmic ray energy spectrum¹⁹.

Persistence is the property of a parameter to remain unchanged for a period of time—if it is warm today it is likely to be warm tomorrow. With persistence, data in a time-series are not fully independent and this affects the degrees of freedom

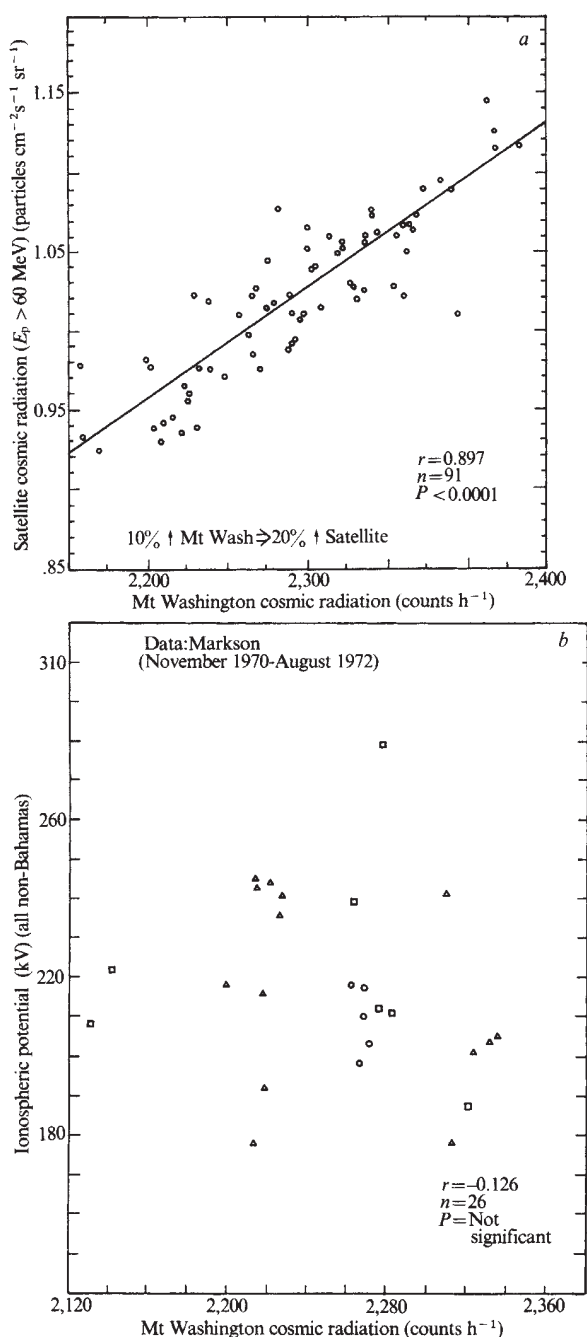


Fig. 2 *a*, Comparison of simultaneous cosmic radiation measurements obtained by the IMP 4 satellite in Earth orbit versus Mount Washington neutron monitor counts of secondary cosmic radiation at the Earth's surface. The period of these data is December 1971–March 1972. *b*, Scatter diagrams of all non-Bahamian V_1 soundings obtained in the aircraft ionospheric potential investigation versus Mount Washington cosmic radiation. □, New York State and Cape Cod (November–December 1970); ○, Maryland (January 1972); ▲, Nova Scotia and New York State (April, July, August 1972).

used in computing significance. There have been too few measurements of the temporal variation of V_1 to estimate accurately the persistence of this parameter, but from inspection of these records the coherency interval seems to be of the order of hours to a day. Assuming that magnetic discontinuities structured by the solar wind modulate cosmic radiation, and analysis of solar wind variations are applicable to cosmic radiation analysis, one can follow the approach of Gosling and Bame²⁰ who suggest that solar wind velocities a minimum of 2 days apart are reasonably independent of each other. Therefore, all the Bahamas V_1 and corresponding cosmic radiation data were

separated into groups that were at least 2 days apart (generally longer) and averaged. For the Bahamas measurements, Fig. 3*a* depicts a correlation of 0.486 with the Mount Washington cosmic rays and Fig. 3*b* shows a correlation of 0.663 when the satellite counts are used; these are significant at about the 0.05 level. For the larger German V_1 data set, also separated into groups a minimum of 2 days apart, Fig. 3*c* shows a correlation of 0.402 with the Mount Washington data and Fig. 3*d* indicates a correlation of 0.367 for the satellite cosmic rays; the respective significance levels are 0.0001 and 0.01. Thus, analyses of two independent data sets, one obtained at relatively frequent intervals during a 3-month period in the Bahamas and the other obtained relatively infrequently over a 17-yr period in Germany, indicate that the Earth's electric field intensity is directly correlated with the rate of galactic cosmic radiation.

The importance of upper atmosphere electrical generators in modulating the Earth's electric field has been discussed elsewhere^{21,22}. To investigate this the B_z component of the interplanetary magnetic field and the auroral (AE) geomagnetic index were correlated with the Bahamas ionospheric potentials. The former is highly correlated with magnetospheric-ionospheric coupled electric fields and the latter is a measure of the auroral electrojet which is maintained by the magnetospheric-ionospheric generator. Figure 4*a* is the scatter diagram for the B_z versus V_1 analysis and Fig. 4*b* shows the relationship of the AE index to V_1 . Both B_z and the AE index are uncorrelated with the same V_1 data set that is well correlated with cosmic radiation.

Considering differences between the analyses, Fig. 1*a, b* for the Bahamas data shows that a 10% increase in cosmic radiation is associated with almost twice the increase in V_1 as the same change in Mount Washington cosmic radiation. This may be explained by the correlation between the satellite and Mount Washington count rates (Fig. 2*a*) which exhibit a 2:1 ratio, that is, a 10% increase in neutron monitor rate would be equivalent to a 20% increase at the Earth orbiting satellite. However, comparison of Fig. 1*c* and *d* for the German data shows the magnitude of the V_1 response is greater for the ground level measurements than for those at the satellite. Furthermore, comparing Fig. 1*a* and *c* the regression line slope for the German data is about half that of the Bahamas data when one correlates these V_1 variations relative to the neutron monitor measurements. These discrepancies are probably due to the greatly different time span of the Bahamas data (3 months) compared with the German data (17 yr) compounded by the differences in the periods of the satellite record (3.5 yr) versus the Mount Washington record (17 yr). It is noted that during the 2-month period when the Bahamas V_1 and satellite cosmic rays could be compared (Fig. 1*b*) the maximum value of cosmic ray flux was only 20% greater than its minimum value, while for the corresponding German data (Fig. 1*d*) the cosmic ray increase was 110%; the larger range of cosmic radiation variation would decrease the slope of the regression line. The different responses between the analyses is probably a function of the period length and times when the measurements were made. The cosmic ray energy spectrum is known to change in complicated ways through the sunspot cycle. This is especially true for the less energetic cosmic ray particles which are capable of ionizing the stratosphere and affect the current flow from the global thunderstorm generator to the ionosphere.

Figure 2*b* shows the critical importance of location when making V_1 soundings. When sources of error associated with convection and aerosol distributions in the exchange layer were identified in the early phases of the aircraft measuring programme, it was decided to move the experimental area to the tradewind region of the Bahamas¹⁷. Here working in a clean maritime air mass that had travelled thousands of miles across the ocean gave measurements that were representative of global atmospheric electrical conditions.

Figure 3 shows the correlations of grouped V_1 and cosmic radiation for both V_1 and the two cosmic ray data sets. These groupings were done to achieve data independence. Each analysis shows that V_1 and cosmic radiation are positively cor-

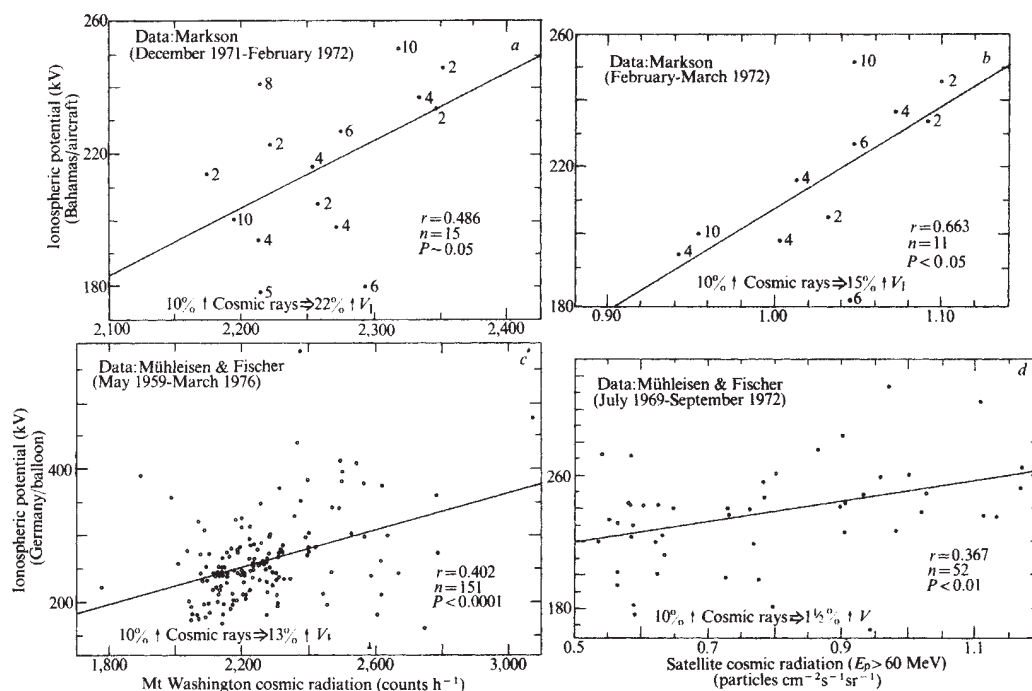


Fig. 3 Scatter diagrams of average ionospheric potentials separated into groups at least 2 days apart compared with corresponding cosmic radiation rates treated in the same manner. The numbers next to the dots give the number of data points used. *a*, Bahamian V_1 data versus Mount Washington cosmic radiation. *b*, Bahamian V_1 data versus satellite cosmic radiation. *c*, German V_1 data versus Mount Washington cosmic radiation. *d*, German V_1 data versus satellite cosmic radiation. These analyses of grouped data were performed to insure data independence.

related. The most statistically significant correlation is seen in Fig. 3c where the relatively large data set of Mühleisen and Fischer could be divided into groups separated by at least 2 days and still have 151 points.

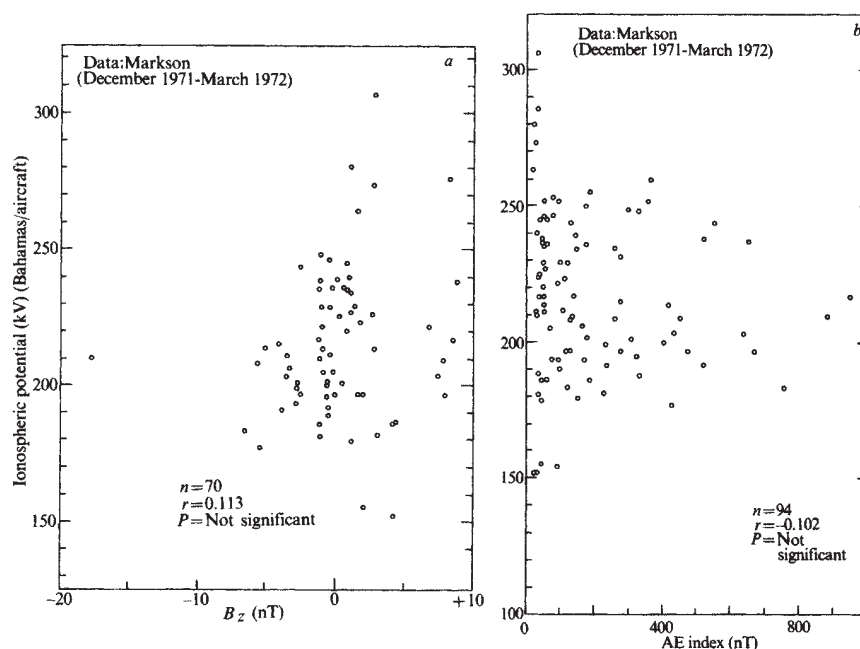
The per cent increases in V_1 associated with 10% increases in cosmic radiation should not be interpreted as quantitative and generalized results but rather as estimates of the range of responses that can be expected. Taken together the various correlations suggest that the ratio of ionospheric potential change to cosmic ray change is greater than unity.

Figures 4a, b which shows scatter diagrams of the relationships of B_z and the AE index to V_1 , have a different appearance

compared with the previous figures which showed the correlation between cosmic radiation and V_1 . These parameters which are a measure of the upper atmosphere generator are uncorrelated with V_1 . The correlations are not significant even though B_z and the AE index were treated as if they were independent data, which is not the case.

We conclude that solar modulation of the Earth's electric field takes place through regulation of ionizing radiation by magnetic discontinuities within the solar wind. Because galactic cosmic radiation is generally the sole agent ionizing the stratosphere and most of the troposphere (essentially all of the latter over the ocean), the Sun controls atmospheric electrification mostly

Fig. 4 Scatter diagrams of parameters representative of the magnetospheric-ionospheric electrical generator compared with V_1 . *a*, Bahamian V_1 data versus the B_z component of the interplanetary magnetic field. *b*, Bahamian V_1 data versus the AE geomagnetic index.



through modulation of galactic cosmic radiation. However, for relatively short periods (hours to a day) after some solar flares, energetic solar particles can be the dominant ionizing source in the stratosphere²³⁻²⁶. In either case, greater ionization in the lower stratosphere would enhance thunderstorm currents and thus the fair-weather electric field. This conclusion is consistent with all past studies of the relationship of solar activity to fair-weather electric field intensity which show (1) a negative correlation between the sunspot cycle and V_I , that is a positive correlation between galactic cosmic radiation and V_I (ref 27), and (2) a positive correlation between solar flares and V_I (refs 9, 10). To examine effects due to solar activity further, I stratified the German V_I data into the years of low sunspot number (high cosmic radiation) and high sunspot number (low cosmic radiation) for comparison. The high cosmic radiation periods were 1962–66 and 1973–76 while the low cosmic radiation periods were 1959–61 and 1967–72. For the high cosmic radiation years the average V_I was 295 kV ($n = 108$); for the low cosmic radiation years the average V_I was 238 kV ($n = 142$). These data thus show a 22% peak-to-peak variation around a mean of 266 kV in phase with the solar cycle cosmic ray variation. During the cycle in question the Mount Washington neutron monitor recorded an ~17% variation around its mean.

A positive correlation between ionizing radiation and V_I implies that solar control of the global circuit takes place in the charging part of the circuit, that is, in the resistive element between the global thunderstorm generator and the ionosphere, and possibly in this generator itself. The effect of ionizing radiation on the return conduction current in the fair-weather part of the circuit would be to short out this resistive element partially and lower V_I . As increased ionization causes an increase in V_I , the effect must be due to amplification of the current output of existing thunderstorms plus possibly positive feedback in the form of increasing the number and electrification of thunderclouds. These findings support a proposed mechanism by which solar variability can modulate atmospheric electrification⁶.

I thank Professor R. P. Mühleisen and Dr H.-J. Fischer for their list of ionospheric potential soundings. M. A. Shea and D. F. Smart provided the edited satellite and Mount Washington cosmic ray data. This work was supported under Office of Naval Research contract no. N00014-79-C-0399.

Received 11 December 1980; accepted 27 February 1981.

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Neutron-capture nucleosynthesis of nature's rarest stable isotope

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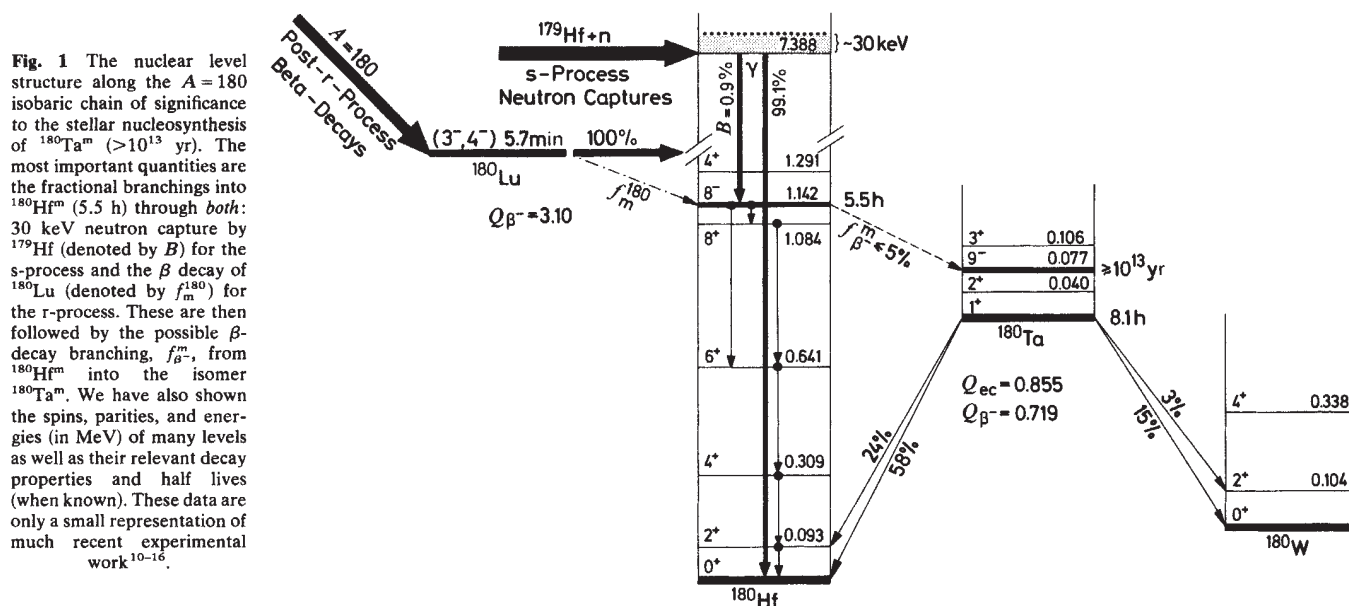
Many attempts¹⁻⁶ have been made to explain the solar abundances of the stable, heavy odd-odd nuclei: ⁵⁰V, ¹³⁸La, ¹⁷⁶Lu and ¹⁸⁰Ta. Among these, ¹⁸⁰Ta (actually an isomeric state, ¹⁸⁰Ta^m) has the distinction of being the rarest, stable naturally occurring isotope (0.012% of all Ta) with a solar abundance⁷ of only 2.46×10^{-6} (per 10^6 Si atoms) and a half life of $>10^{13}$ yr against β decay. Although the variety of mechanisms previously proposed can in some special cases account adequately for the small abundance of ¹⁸⁰Ta^m, it is difficult to test quantitatively those special types of synthesis invented only to explain such a small number of nuclei. In contrast, we show here from our experimental work that ¹⁸⁰Ta^m can also be accounted for by relatively small branchings in the much more common slow (s)- and rapid (r)-processes of stellar nucleosynthesis by neutron capture.

Because of its small abundance, the nucleus ¹⁸⁰Ta^m does not fit into the general systematics of s-, r- and p-process nuclei. This has been confirmed especially by recent p-process calculations^{8,9} which cannot reproduce the solar abundance of this odd-odd nucleus because of the ease with which it is destroyed by photodisintegration reactions at explosive stellar temperatures. We have attempted to incorporate ¹⁸⁰Ta^m into the systematics of s- and r-process nuclei. Figure 1 shows nuclear structural information for the neutron-capture and β -decay processes that affect the production of ¹⁸⁰Ta^m. Most of the information is taken from the most recent compilation in *Table of Isotopes*¹⁰, except for the structure of ¹⁸⁰Ta which reflects recent and much-needed experimental work¹¹⁻¹⁵ on its properties. This work has not only identified many low-lying levels in ¹⁸⁰Ta but has also finally clarified which of the long-lived species in ¹⁸⁰Ta is the isomer and which is the ground state. Furthermore, the identification¹³ of the 9^- nature of ¹⁸⁰Ta^m is very important for present considerations.

Figure 1 reveals that the key to our arguments is the population of the long-lived 8^- isomeric state ¹⁸⁰Hf^m (5.5 h). The possible¹⁶ fractional β decay, $f_{\beta^-}^m$, of this level then becomes the final branch in the nuclear flow that leads directly to ¹⁸⁰Ta^m. The initial population of ¹⁸⁰Hf^m in turn depends on which neutron-capture process of nucleosynthesis we are considering. If we examine the s-process neutron-capture path that flows through the valley of β stability, we see from Fig. 1 that it leads along the Hf isotopic chain through ¹⁷⁹Hf to the population of ¹⁸⁰Hf^m. In the γ cascades down through the ¹⁸⁰Hf compound nucleus, a fraction, B , of the decays is temporarily arrested at the 5.5-h isomer. However, for the r-process, the initial flow into ¹⁸⁰Hf is somewhat different. As this process occurs far on the neutron-rich side of the valley of β stability, it is the subsequent β -decay branching at ¹⁸⁰Lu along the $A = 180$ isobaric chain that determines any r-process flow into ¹⁸⁰Hf^m.

Considering first the possible s-process contribution, we note that the total s-process yield at $A = 180$ is usually expressed in the form of cross-section $\times$ abundance as: $\sigma N_s(A = 180) = 5.56$ (mb $\times$ Si $= 10^6$) according to a recent study¹⁷ of s-process systematics at 30 keV. Therefore, the nuclear flows in Fig. 1 show that:

$$\begin{aligned} N_s(^{180}\text{Ta}^m)/N_\odot(^{180}\text{Ta}^m) &= B\sigma N_s(A = 180)f_{\beta^-}^m/(\sigma(^{180}\text{Ta}^m)N_\odot(^{180}\text{Ta}^m)) \quad (1) \\ &= 2.26 \times 10^6 B f_{\beta^-}^m/\sigma(^{180}\text{Ta}^m) \end{aligned}$$



for a steady flow of the neutron-capture current through $\sigma(^{180}\text{Ta}^m)$, the 30-keV neutron-capture cross-section of $^{180}\text{Ta}^m$. Unfortunately, until we have an experimental determination, we must use the Hauser-Feshbach formalism¹⁸ to calculate $\sigma(^{180}\text{Ta}^m)$. Taking into account the new information¹³ that it is a 9^- level, we obtain $\sigma(^{180}\text{Ta}^m) = 1,820$ mb at 30 keV and note that this value may be uncertain by a factor of two.

As experimental input to the numerical evaluation of equation (1), we have measured the cross-section for $^{179}\text{Hf}(n, \gamma)^{180}\text{Hf}^m$ with the activation technique. Neutrons were generated by the $^7\text{Li}(p, n)$ reaction at ~ 25 keV above threshold. This gives a neutron distribution in a forward cone of 120° that is very similar to a maxwellian at 25 keV thermal energy. A metallic Hf foil of natural isotopic composition back-to-back with an Au foil as a cross-section standard were both irradiated with this neutron spectrum. The induced activity was then counted using a characteristic γ -ray line with a Ge(Li)-detector. The activation foils all had a diameter of 6 mm, and the sample characteristics are summarized in Table 1. Additionally, Table 2 shows the decay properties and our measurements of the cross-sections. More details about the experimental technique can be found elsewhere⁷. The maxwellian-averaged Au cross-section was calculated from the ENDF/B-IV microscopic data file, and the measured Hf cross-section was extrapolated to $kT = 30$ keV using a theoretical calculation of Benzi *et al.*¹⁹. At 30 keV, the total capture cross-section of ^{179}Hf was evaluated²⁰ to be 1,346 mb, so we find that $B(30 \text{ keV}) = 0.90\%$, in contrast to its thermal value²¹: $B(0.025 \text{ eV}) = 0.75\%$.

With regard to possible r-process contributions, the branchings in Fig. 1 show that:

$$\begin{aligned} N_r(^{180}\text{Ta}^m)/N_\odot(^{180}\text{Ta}^m) &= N_r(A=180)f_m^{180}f_\beta^{180}/N_\odot(^{180}\text{Ta}^m) \\ &= 1.22 \times 10^4 f_m^{180}f_\beta^{180} \end{aligned} \quad (2)$$

where f_m^{180} is the branching ratio of 5.7 min ^{180}Lu to $^{180}\text{Hf}^m$. This branching ratio f_m^{180} could be a β -decay from ^{180}Lu directly to the 8^- isomer and/or β decays to higher-lying levels in ^{180}Hf followed by γ transitions to $^{180}\text{Hf}^m$. For the numerical results given above, we have used $N_r(A=180) \approx 3 \times 10^{-2}$ ($\text{Si} \approx 10^6$) as being representative¹⁷ of the average r-process yield along the $A=180$ isobar.

Table 1 Sample characteristics

Sample	Chemical	Isotopic	Investigated isotope	Thickness (atoms per barr)
Hf	Metal	Natural	^{179}Hf	8.04×10^{-5}
Au	Metal	—	^{197}Au	2.00×10^{-4}

Table 2 Decay parameters and cross-sections

Reaction	$T_{1/2}$	$E_\gamma(\text{keV})$	$I_\gamma(\%)$	$\sigma(\text{mb})^*$	
				$kT=25 \text{ keV}$	$kT=30 \text{ keV}$
$^{179}\text{Hf}(n, \gamma)^{180}\text{Hf}^m$	5.5 h	332	94.4 ± 0.2	13.5 ± 0.6	12.2 ± 0.6
$^{197}\text{Au}(n, \gamma)^{198}\text{Au}$	2.69 days	412	95.52 ± 0.06	672.4 ± 16.8	—

*Quoted uncertainties are calculated from: Au standard (2.5%); decay scheme (0.2%); Ge(Li) efficiency (1.5%); counting statistics (1%); divergence of neutron beam (1%); others (3%).

To complete the evaluation of both equations (1) and (2) we need to determine the value of f_β^{180} . From a theoretical point of view, the β^- energetics of the situation in Fig. 1 show that the $^{180}\text{Hf}^m \rightarrow ^{180}\text{Ta}^m$ β decay has an endpoint energy of 210 keV and is an allowed Gamow-Teller transition because no parity change is involved. The decay would therefore be expected²² to be characterized by an average $\log ft$ value in the range: $\langle \log ft \rangle = 5.7 \pm 1.1$. Using tabulated²³ f -values for such a decay, one then obtains an estimate of:

$$0.14\% \leq f_\beta^{180} \leq 22\% \quad (3)$$

with a value of $f_\beta^{180} \leq 3.8\%$ corresponding to the limit given by Gallagher *et al.*¹⁶ of $\log ft \geq 5.3$.

We can now make an approximate evaluation of equation (1) using the values: $B = 0.90\%$, $f_\beta^{180} \approx 3.8\%$, and $\sigma(^{180}\text{Ta}^m) = 1,820$ mb to obtain

$$N_s(^{180}\text{Ta}^m)/N_\odot(^{180}\text{Ta}^m) \approx 0.42 \quad (4)$$

Furthermore, using $f_\beta^{180} = 3.8\%$, we see from equation (2) that a branching ratio as small as $f_m^{180} = 0.2\%$ can produce the entire solar abundance of $^{180}\text{Ta}^m$ through the r-process.

Considering how close to unity these estimates are for the production ratios for $^{180}\text{Ta}^m$, we believe that there is ample motivation for measurements (or remeasurements) of the following important quantities needed in equations (1) and (2): (1) the β -decay branching of 5.5-h $^{180}\text{Hf}^m$. For this purpose, highly enriched ^{179}Hf is needed; (2) the neutron-capture cross-section of $^{180}\text{Ta}^m$ at 30 keV; (3) the total cross-section of $^{179}\text{Hf}(n, \gamma)^{180}\text{Hf}$ at 30 keV. As the cross-section is large, a sample of less than 1 g is probably sufficient; (4) a firm upper limit on the fraction of the total $A=180$ isobaric β decays that yield $^{180}\text{Hf}^m$. This is a sensitive problem in nuclear chemistry and radiochemical milking; and (5) the detailed level structure of ^{180}Ta up to a few hundred keV.

Once these experimental improvements are available, the numerical evaluation of our arguments for the neutron-capture origin of $^{180}\text{Ta}^m$ will be on much firmer ground.

Received 6 February; accepted 25 March 1981.

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Formation of helium platelets in molybdenum

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Over the past two decades, helium introduced into metals either by ion implantation or by the (n, α) reaction has been studied over a wide range of concentrations and temperatures^{1,2}. Early studies were relevant to fission reactor technology^{3,4} but more recent emphasis has been on the role of helium in fusion reactor environments. One area of interest concerns the interaction of helium ions from the plasma with the first-wall surface while the influence of the helium from the (n, α) reaction on void nucleation, and on grain boundary bubble nucleation, is also under investigation⁵. One long-established property of insoluble helium and other inert gases in metals is their tendency to precipitate as bubbles. However, the inference that inert gas clusters always assume a three-dimensional form may not be justified. We present here experimental evidence showing that, at least in molybdenum, helium can initially aggregate in an unexpected planar form. Furthermore, we have found a bubble nucleation route involving the formation of several small bubbles from a single helium platelet.

At temperatures below $0.3 T_m$, where T_m is the melting point, the injection of helium in molybdenum and several other metals, leads to ultrahigh density ($\sim 10^{25}$ per m^3) bubble formation⁶ and bubble lattices are frequently observed⁶⁻⁸. However, bubble observations by transmission electron microscopy (TEM) can normally only be made at helium concentrations exceeding ~ 5 atomic %; at lower helium levels the high bubble and dislocation densities severely impede any attempts to study the early stages of bubble growth. Fortunately, a method to limit the normal high density of nucleation centres has been introduced

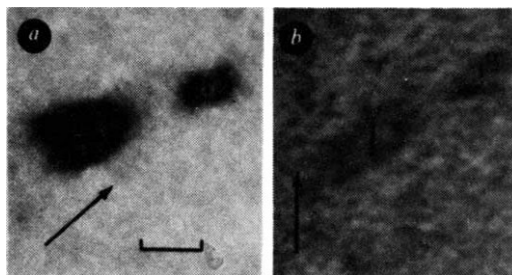


Fig. 1 Transmission electron micrographs with a beam direction close to $[001]$ showing the edge-on projection of two helium platelets lying on $\{110\}$ planes. The direction of the operating reflection, g , is denoted by the arrows. a , $g = [020]$; the platelet and loop images are visible; b , $g = [\bar{1}10]$; the loop image is out of contrast. Scale bar, 15 nm.

using thermal helium desorption spectrometry (THDS)^{9,10}. The THDS technique in its simplest form provides information on the binding of helium to traps in metals by first depositing energetic helium ions near to the metal surface and then examining desorption peaks during a temperature ramp. In the procedural variation of THDS which we consider here the metal is first injected with a low dose of 3 keV helium ions (typically 10^{16} ions m^{-2}) and then annealed to 1,050 K. This procedure results in a known concentration of HeV defects (monovacancies containing one helium atom) which then act as unsaturable traps for a further dose of low energy non-damaging 150 eV helium ions. In this way, no additional nucleation centres are formed but each initial HeV trap can acquire a controlled number of helium atoms up to several thousand. The unsaturable nature of the traps stems from a continuation of the initial trap mutation process¹¹ in which the combined strain of several helium atoms at a monovacant site can result in the removal of a lattice atom to an interstitial position leaving the helium atoms in a divacancy. Thus, as further helium is captured the trap size will expand appropriately. We have taken advantage of the control on the size and density of helium clusters inherent in the above approach, to prepare THDS samples suitable for subsequent TEM examination.

So far, five specimens have been prepared by this technique with a variety of initial trapping sites and different degrees of helium filling from 400 up to 4,000 helium atoms per trap. The specimens were in the form of 3-mm disks of single crystal zone-refined molybdenum with surface normals close to $\{001\}$. A back-thinning electropolishing technique was used to obtain suitable electron thin regions in the surface layers of interest. On examination by TEM, using a Philips EM400T microscope at 120 keV, all specimens contained strain contrast loop images. In the thinner regions of foils (20–60 nm) the areal density of images was independent of thickness and matched the variation of initial trapping site density. Also the size of these strain images reflected the degree of helium trapping. Thus, they could be confidently identified with the helium traps detected by THDS. In the thicker regions of the foil, there was an increase in areal image density due to loops not associated with the traps. We anticipated the helium trap images would be due to strain fields around small over-pressurized helium bubbles; instead it became clear in three specimens that the loop images were around helium aggregates having an unexpected platelet character. The platelets lay on $\{110\}$ planes and $g \cdot b = 0$ criteria (where g is the operating reflection and b is the Burgers vector) showed the loops to have Burgers vectors orthogonal to their platelets. The character of the platelets was most easily seen when they were viewed close to edge-on; two such platelets are shown in Fig. 1a and b where the beam direction was close to $[001]$. In Fig. 1a with $g = [020]$, the platelets are seen as a single dark line surrounded by the loop image while in Fig. 1b with $g = [\bar{1}10]$ the latter image is removed ($g \cdot b = 0$) to leave only the

platelets visible. When viewed with the platelets inclined to the beam, the images become more complex with an inner image associated with the platelet together with the outer loop contrast. Several such images are seen in Fig. 2.

The observed platelets can be described as disk-shaped cavities containing helium. From the number of helium atoms per trap, the thickness of the platelet images (~ 1 nm) and their diameter, we can estimate that there are between two and three helium atoms per vacancy in the cavity. The atomic densities of helium thus seem to be $>10^{29}$ atoms m^{-3} . Similar high values have been estimated by Donnelly *et al.*¹² during optical studies of helium bubbles in aluminium. The gas pressures in the platelets are clearly immense and give rise to strain fields, and contrast effects, similar to those from intrinsic interstitial dislocation loops. The platelets can be considered analogous to the disks of helium seen in boron carbide after neutron irradiation¹³⁻¹⁵, although the mechanics of nucleation could well be different in the different host lattice structures. More relevant may be the report of diffraction pattern streaking, attributed to argon-vacancy platelets, found in copper after bombardment with argon ions¹⁶.

The effect of temperature on the platelets was followed by *in situ* microscope annealing. Between 600 and 800 °C the platelets transformed into helium bubbles (Fig. 3). Again, an unforeseen result emerged; the gas from a platelet did not minimize its surface energy by forming a single bubble but instead formed a cluster of small bubbles. We have mentioned that platelet formation was found only in three specimens; in the other two specimens, one with a very high initial density of helium traps and the other where each trap contained $\sim 4,000$ helium atoms, separated clusters of small bubbles were found, even without annealing. The similarity between the bubble clusters in these two specimens and those in the annealed samples was close enough to be fairly certain that they too had evolved from platelets. The mechanism for the platelet to bubble cluster transformation at room temperature is uncertain. It is possible that the interaction of lattice strains from different platelets could play a part but further studies are required to establish the critical trap density and helium filling parameters.

It is interesting that the size of individual bubbles and their separation within a cluster (~ 3 to 5 nm) are close to those eventually found after conventional implantation to high helium concentrations. We may have uncovered a route that leads to the ultrahigh bubble density, and eventually to the bubble lattice, found in molybdenum and other metals.

Regarding the initial platelet nucleation, by using atomistic calculations with interatomic potentials for α -iron, Caspers *et al.*¹⁷ have demonstrated that two-dimensional clustering of helium-vacancy complexes on $\{011\}$ planes is not unexpected.

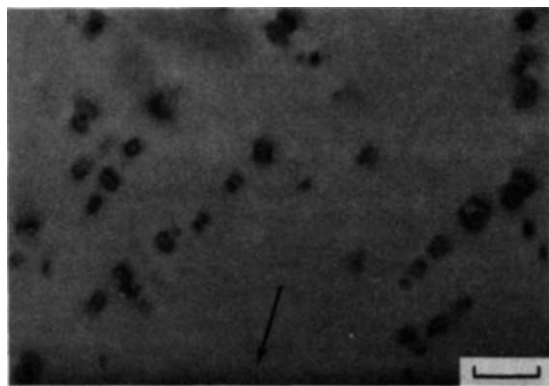


Fig. 2 Micrograph of inclined platelet/loop images using $g=[1\bar{2}1]$ (arrowed) and a beam direction close to $[1\bar{1}3]$. Several complex images can be seen; the simpler loop images arise from the platelet growth process. Scale bar, 40 nm.

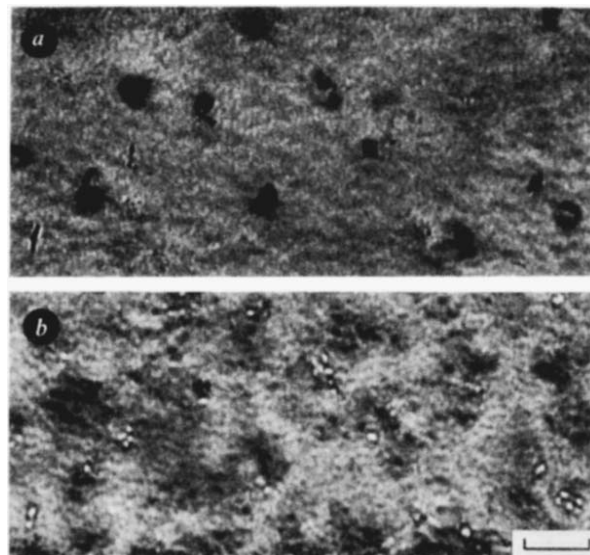


Fig. 3 Effect of annealing on platelet morphology. *a*, Unannealed; *b*, same area after 10 min at 800 °C. The platelets, some edge-on, and some inclined with loop contrast, have transformed to clusters of bubbles. Scale bar, 25 nm.

The results show that the self-interstitials removed from a complex by the trap mutation process do not migrate away to other sinks but remain bound to the complex and thus could have an important role in its morphology. Intermediate stages of platelet growth may not be easy to model but in later stages we have experimental evidence, described in more detail elsewhere¹⁸, which demonstrates that the helium platelets can increase their thickness by cooperatively moving out a plane of host atoms in a glide direction. This is essentially the loop punching process proposed by Greenwood *et al.*¹⁹ for the relief of pressure in gas bubbles. The process gives the bubble, or in our case the platelet, an extra plane of vacancies in exchange for an interstitial loop in the lattice. The characteristic of such loops is their alignment along a glide direction ($\langle 111 \rangle$ in body-centred cubic metals). We found numerous instances of two to four loops aligned along such directions and having $\langle 111 \rangle$ Burgers vectors. The presence of these loops was the reason mentioned earlier for the increase in image density in thicker foil regions. Note that with our $[001]$ specimen orientation all the $\langle 111 \rangle$ glide directions bisected the specimen surfaces. Loop loss to the surface was therefore quite likely and was sometimes observed during microscope observation. To study the loop punching process in more detail, and to establish the platelet size for its initiation, specimens with $\langle 011 \rangle$ normals would be preferred because these have two of the four $\langle 111 \rangle$ directions lying parallel to the specimen surface.

We have uncovered new and unexpected aspects of helium clustering behaviour in molybdenum and demonstrated the effectiveness of combining the THDS and TEM techniques. Some areas requiring further experiments have already been discussed such as the platelet to bubble cluster transformation and the initiation of loop punching—but the work also raises other questions. These include the effect of temperature during helium implantation and whether the platelet formation might be found with other inert gases or in other metals. However, the main interest might be to establish if the platelet formation and the possible route to bubble nucleation still operates when the deposition of helium is accomplished by the displacement damage (and the consequent high density of vacancy traps) characteristic of conventional helium implantation experiments.

We thank K. T. Westerduin for technical assistance. J.H.E. thanks the Physical and Metallurgical Departments of the Technical University, Delft for their hospitality during the initiation of this work.

Received 2 February; accepted 23 March 1981.

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Atmospheric transport of continentally derived lipids to the tropical North Pacific

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Lipid class compounds have been used as source markers to ascertain the terrestrial, marine, or anthropogenic origin of atmospheric aerosols from rural continental and coastal oceanic environments^{1–11}. However, there are only limited data on the composition and source of lipids in remote marine aerosols. Studies off the West African coast suggest that surface waxes of terrestrial vascular plants are a major source of lipid class compounds in dust samples^{6,11}. Uniform concentrations of lipids found^{7,10} in aerosols from urban, rural and oceanic regions imply either a long residence time for continental material or a uniform production rate of the compounds over the oceans. It has also been concluded^{1–3,8} that oceanic aerosols have significant marine sources. However, previous organic chemical studies have not been conducted in conjunction with other chemical analyses of the aerosols or with climatological and meteorological studies. We report here, as part of the Sea/Air Exchange Program (SEAREX), the temporal variation of the transport of continentally-derived lipids (hydrocarbons, fatty alcohols and fatty acids) to the central tropical North Pacific.

Samples were collected during the SEAREX wet and dry season experiments (April–May and July–August 1979) from a 20-m tower erected on the windward shore of Bokandretok Island, Enewetak Atoll, Marshall Islands. The site, 11°20' N, 162°20' E, is located in the north-east tradewind regime, ~5,000 km south-east of Asia and 8,000 km west-southwest of the North American continent. Atmospheric particulate matter samples collected using 20×25 cm Gelman A/E glass fibre filters were pre-extracted once with acetone/MeOH and twice with CH₂Cl₂ and stored in precleaned glass jars. These filters have a collection efficiency of ≥98% for particles with radii >0.015 μm at the 20–60 m³ h⁻¹ flow rate used¹². Filters were mounted in aluminium holders within aluminium rain shelters. To prevent back diffusion or injection of organic material onto the filters, a stainless steel check valve was located between the filter frame and more than 20 feet of aluminium tubing connecting the high volume sampler to the high volume pump.

Polyurethane foam plugs placed behind the filter also prevented back diffusion of organic material¹². Quadruplicate filters were used for most sampling periods, two filters as blanks (one in each of two shelters), and two filters for duplicate samples in these separate shelters. Samples were collected for 2–10 days (2,000–10,000 m³ air sampled). Comparison between high and low loadings showed no statistically significant difference in hydrocarbon (nC_{21} – nC_{36}), fatty alcohol (nC_{13} – nC_{32}) or fatty acid (nC_{13} – nC_{32}) concentrations. Less than 5% of the material collected on the first of back-to-back mounted filters appeared on the second filter. There were no qualitative lipid composition changes between these filters. The collection system was electronically controlled; relevant meteorological parameters were monitored continuously. Collection was stopped automatically when the surface wind direction was not from the open sea (040°–160°), when the wind speed dropped below 5 knots, when the condensation nuclei count exceeded 750 particle cm⁻³ or when precipitation started. Our sampling system has apparently been free of contamination from anthropogenic sources on the nearby main island of Enewetak, as no single polynuclear aromatic hydrocarbon (PAH) was found at concentrations

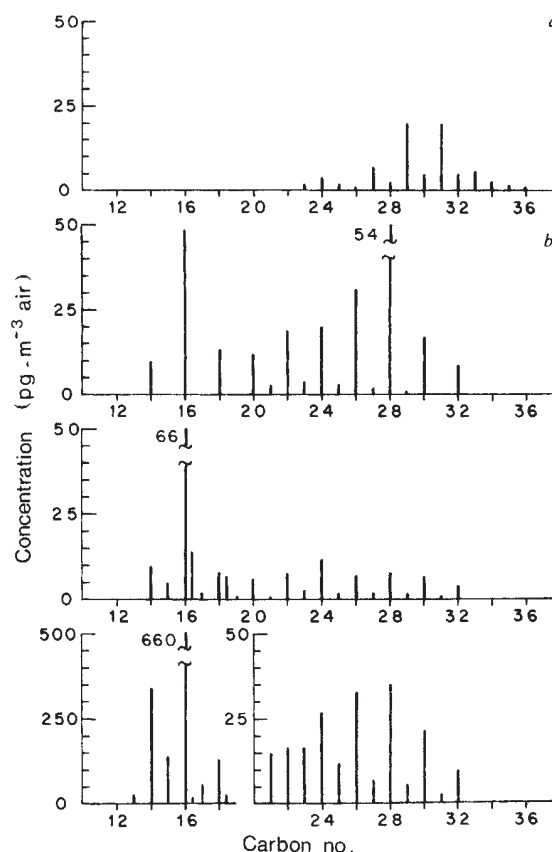


Fig. 1 Concentrations of individual aliphatic hydrocarbons (a), fatty alcohols (b), fatty acid esters (c) and fatty acid salts (d) as a function of carbon number in a marine aerosol sample collected at Enewetak Atoll, 17 May 1979. Sample volume was 4,709 m³ collected at 75 m³ h⁻¹. Peaks at half carbon numbers represent monounsaturated compounds. Detection limits for the lipid classes are 2 pg m⁻³ air in a 1,000 m³ air sample. Blanks were run for each sample as described in the text. These values were subtracted from the samples. For the alkanes, the blank levels were 5–10 times lower in concentration (~5 pg m⁻³ for 5,000 m³ sample) than the sample and show no odd/even carbon predominance. The fatty alcohol blanks for compounds >C₂₁ were <1 pg m⁻³ per component. Blanks for compounds C₁₂–C₂₁ were less than five times the sample concentration (5 pg m⁻³ for a 5,000 m³ sample) except for C_{16:0} which was ~15 pg m⁻³. Fatty acid ester blanks were <1 pg m⁻³ for compounds >C₂₁. C_{14:0}, C_{16:0}, C_{18:0} were less than 15 pg m⁻³. Fatty acid salt blanks averaged <5 pg m⁻³ for components >C₂₁. C_{14:0}, C_{16:0}, C_{18:0} had blanks as high as 100 pg m⁻³ but were ~3 times lower in concentration than found in the samples.

Table 1 Atmospheric aerosol concentrations of aliphatic hydrocarbons, fatty alcohols, fatty acid esters and fatty acid salts from samples collected at Enewetak Atoll, April–August 1979

Mid-date Volume (m ³)	18 April 5,300	6 May 4,800	17 May 4,700	5 July 4,500	14 July 8,500	29 July 9,200
Aliphatic hydrocarbons						
C ₂₁ –C ₃₆ *	160	63	79	72	23	20
CPI† (C ₂₁ –C ₃₆)	3.2	2.2	2.8	3.8	2.5	4.0
Major components‡	29, 31, 27	31, 29, 33	29, 31, 27	29, 31, 27	29, 31, 33	31, 29, 27
Fatty Alcohols						
C ₁₃ –C ₂₀ *	4	2	85	—	25	12
Major components‡	20, 16	20, 14	16, 18, 20	—	16, 18, 14	16, 14, 20
C ₂₁ –C ₃₂ *	170	72	160	210	58	60
Major components‡	28, 26, 30	28, 30, 32	28, 26, 24	30, 28, 32	30, 28, 26	30, 28, 32
CPI† (C ₂₁ –C ₃₂)	8.6	12	12	15	21	14
Fatty acid esters						
C ₁₃ –C ₂₀ *		290	120	90	59	34
Major components‡		16, 18, 14	16, 16:1, 14	16, 14, 18	16, 14, 18	16, 14, 15
C ₂₁ –C ₃₂ *		91	56	75	10	6
Major components‡		24, 22, 26	24, 22, 28	24, 22, 26	24, 22, 26	24, 20, 22
CPI† (C ₂₁ –C ₃₂)		6.4	4.5	7.1	7.3	12
Fatty acid salts						
C ₁₃ –C ₂₀ *	3,200	4,000	1,400	87	730	1,400
Major components‡	16, 14, 15	16, 14, 18	16, 14, 15	14, 18:1, 16:1	16, 14, 18	16, 14, 15
C ₂₁ –C ₃₂ *	670	250	200	140	36	53
Major components‡	28, 22, 30	28, 24, 30	28, 26, 24	28, 30, 26	30, 22, 24	26, 22, 24
CPI† (C ₂₁ –C ₃₂)	2.6	1.8	2.4	4.0	4.1	>10

*Concentrations are in pg m⁻³ air.

†Carbon number in order of decreasing abundance.

‡CPI, carbon preference index. Odd/even for aliphatic hydrocarbons, even/odd for fatty alcohols and acids.

>5 pg m⁻³ (see ref. 9 for an example of PAH aerosol values from urban, rural and seashore areas). No insects were detected on the filters and no branched hydrocarbons (>C₂₅), indicative of insect cuticles¹³, were detected on our filters (Fig. 1), suggesting that contamination from insects trapped on the filter was minimal.

After sample collection, the filters were spiked with hydrocarbon, fatty alcohol and fatty acid internal standards for recovery determinations, and were solvent extracted with 400 ml of glass-distilled CH₂Cl₂ for 16–20 h. (Extraction of the filter with another 400 ml portion of CH₂Cl₂ yielded <1% of the hydrocarbons and fatty alcohols found from the first extraction.) The CH₂Cl₂ extract was evaporated to dryness under vacuum at <25 °C. An aliquot of the extract was separated into individual compound classes by adsorption chromatography by elution with a series of solvents of increasing polarity (ranging from hexane to 50/50 hexane/toluene to 20% ethyl acetate in hexane) to separate the lipids into alkane and alkene, polynuclear aromatic hydrocarbon, fatty acid ester, fatty alcohol, and steroid alcohol fractions. The alcohols were converted to their acetates with acetic anhydride in pyridine. The individual fractions were spiked with appropriate external standards for quantitative determinations and analysed by high resolution glass capillary gas chromatography (GC) using a deactivated (by persilylation) SE-52 coated (20.2 m × 0.32 mm i.d.) column¹⁴. Compound structures were identified by comparison of GC retention times and mass spectra with authentic standards.

Another aliquot of the CH₂Cl₂ extract was treated with BF₃/MeOH to convert fatty acid esters (wax esters and triglycerols) into their methyl esters (free fatty acids remained on the filter as salts). The transesterified aliquot was then treated as described above. After methylene chloride extraction, the filter was treated with 0.1 M HCl/MeOH to protonate all weak acid salts. The acidic MeOH was then extracted with hexane and the liberated fatty acids were converted to their corresponding methyl esters and analysed as described above.

Aliphatic hydrocarbons, fatty alcohols, fatty acid ester and fatty acid salt concentrations in pg m⁻³ are listed in Table 1 for six sampling periods throughout the five-month Enewetak experiment. Concentrations of the individual members of the lipid classes and their compound distributions are shown in Fig.

1 for one sample taken at the end of the dry season. Total aliphatic hydrocarbon (C₂₁–C₃₆) concentrations range from 20 to 160 pg m⁻³ (~3% of the total CH₂Cl₂ extract). An odd-to-even carbon preference index of 2.2–4.0 and the major alkanes being *n*C₂₇, *n*C₂₉ and *n*C₃₁ strongly suggest vascular plant wax alkane inputs^{15,16}. Only traces of aliphatic hydrocarbons <C₂₀ were detected, possibly due to loss from the filter of these more volatile compounds. No indication of any 'hump' from fossil fuel material was observed in the gas chromatograms of the hydrocarbon fractions^{12,17}. Very low levels (<5 pg m⁻³) of >C₂₀ *n*-alkenes or branched alkanes were found. The increased reactivity of alkenes relative to alkanes towards photolytic or chemical oxidation may account for these low levels.

The total fatty alcohol (C₁₃–C₃₂) concentrations range from 72 to 245 pg m⁻³ (~9% of the total CH₂Cl₂ extract). The very strong even-to-odd carbon preference index of 8.6–21 for fatty alcohols >C₁₉ and the presence of major amounts of *n*C₂₆, *n*C₂₈ and *n*C₃₀ compounds again suggest higher plant waxes as a source of the aerosol lipids¹⁶. Only a few peaks of low concentration were attributable to unsaturated fatty alcohols or fatty alcohols derived from marine sources such as C_{12:0}, C_{16:0}, C_{16:1} and C_{18:1} (in C_{x:y} *x* = number of carbons and *y* = number of double bonds). No major (<20%) difference was observed in fatty alcohol concentration when the BF₃/MeOH treated CH₂Cl₂ extract (before column chromatography) was compared with the untreated extract. Hence, few *n*-alcohols were present in the form of wax esters. As BF₃/MeOH is known to destroy some unsaturated fatty alcohols such as phytol, we cannot rule out the possibility that unsaturated *n*-alcohols were esterified as waxes. Low concentrations (<10 pg m⁻³) of steroid alcohols were detected; cholesterol was the major component.

Total esters (wax esters, triglycerols, and so on) of C₁₃–C₃₂ fatty acids range from 40 to 381 pg m⁻³ (~7% of the total CH₂Cl₂ extract), whereas the fatty acid salts have a range of 227–4,250 pg m⁻³ (>50% of the total acidic MeOH/hexane extract). Both fractions exhibit high even-to-odd carbon preference indices for C₂₁–C₃₂. Major components for both fatty acid esters and salts >C₁₉ are *n*C_{22:0}, *n*C_{24:0}, *n*C_{26:0} and *n*C_{28:0} again indicative of a natural terrestrial source¹⁶. However, the fatty acid fractions also have high concentrations of lower carbon number homologues (C₁₃–C₂₂). These compounds are

most likely from a marine source ($C_{14:0}$, $C_{16:0}$, $C_{16:1}$, and $C_{18:1}$)¹⁸, although terrestrial plant wax bacteria, present on plant wax surfaces, also contain these components¹⁹⁻²¹. Marine-derived fatty acid salt and ester (C_{13} – C_{22}) and sea-salt concentrations are lower in July than in April and May (Table 1, ref. 22). This may be due to the 50% decrease in mean wind speed in July relative to the springtime.

The lower molecular weight portion of the gas chromatogram for the aliphatic hydrocarbons and fatty alcohols had few peaks other than those described above. However, several unidentified low molecular weight fatty acids (C_{13} – C_{22}) were present in addition to the saturated n -fatty acids. The structures of these compounds await GC-MS elucidation, although it has been determined that polyunsaturated acids are <20% in either fraction. Again, the low level of unsaturated compounds is not surprising considering their oxidative and photochemical lability relative to their saturated counterparts. The fatty acid results from Table 1 show that in many cases the salts are >10 times higher in concentration than the esters. To our knowledge, these are the first results comparing these two fatty acid fractions from the same samples.

The data shown in Table 1 and Fig. 1 clearly show a strong terrestrial plant surface wax input for the lipids analysed. Finding a strong input of terrestrial plant waxes >5,000 km from a continental source was certainly unexpected. However, mineral dust, carbon isotope and radionuclide studies yielded results explaining both the presence of terrestrial plant surface wax lipids and their decrease with time at the Enewetak station. Duce *et al.*²² used the atmospheric Al concentration as an indicator of continental dust produced from soil and crustal weathering processes. A dramatic decrease of Al concentration occurred from mid-April to early August. Although the concentrations of three lipid class compounds do not follow this decrease exactly, the same general trend is present. The lack of an exact correlation is not surprising because the particles containing these lipids, plant epicuticular waxes, soil or loess, probably have somewhat different terrestrial sources than the dust aluminosilicates. Similarly, the ^{210}Pb activity of the particulates collected during the Enewetak experiment dropped markedly during the five month field program (K. Turekian, personal communication), again roughly in parallel with the total lipid concentrations. ^{210}Pb derives ultimately from a gaseous land source (^{222}Rn) but is formed and attaches to particles in the atmosphere. The rough similarity of these trends can be understood in terms of a fine particle, land-derived source for the organic compounds, transported offshore, initially in a continental (^{222}Rn) air mass. Carbon isotopic studies²³ also showed a continental source for the small size particles (<0.5 μm). These investigators found $\delta^{13}\text{C}$ ratios of –26 to –28‰ for four samples collected during the Enewetak experiment.

The decrease in dust concentration at Enewetak during the five month experiment was rationalized on the basis of the seasonality of dust storm activity in China and the seasonal changes in the large scale wind fields over the Pacific²². This observation of such large amounts of Asian dust during April and May at Enewetak was unexpected because the surface winds are within the easterly (trade) wind regime. However, a strong westerly flow north of 20° N extending from Asia into the central North Pacific is present at 700 mbar (3,000 m). During June–August, the latter part of the Enewetak experiment, conditions were not favourable for the transport of dust to Enewetak due to a decrease in these upper altitude winds²².

The concentrations of the particulate lipid class compounds reported here are, to our knowledge, the lowest yet observed for open ocean areas far from continental influence. Unfortunately the paucity of other work and differences in methodology make precise comparisons with other regions difficult. The most comparable data are those of Hahn and coworkers (refs 7, 10 and personal communication) who found concentrations of individual particulate n -alkanes in the C_{21} – C_{28} region in the range of 200–550 pg m^{-3} with no odd/even carbon preference

for marine aerosol samples collected off the west coast of Ireland. Similar levels were indicated for clean marine air at Cape Grim, Tasmania and in four other samples collected from ships in North Atlantic air masses (refs 7, 10 and J. Hahn, personal communication). Unfortunately, as there are no data for compounds above nC_{28} , any marked odd/even alternation, strongly indicative of land source (Fig. 1a), cannot be discerned.

Fatty alcohol, fatty acid and n -alkane values have been reported for dusts collected using large nets off the West African coast⁶; a several orders of magnitude concentration range was observed. The lower values found, for samples collected in the south-east trades, were in the same concentration range as those found in Enewetak (Table 1), and the individual compound distributions of the lipids were very similar. However, the net dust data are only for large particles (>2 μm diameter) while the Enewetak data are for all particles >0.03 μm diameter. Marty *et al.*⁸ reported much higher aerosol n -alkane values of 30–50 ng m^{-3} and fatty acid values of 80–230 ng m^{-3} from samples collected off north-west Africa on glass fibre filters. Their n -alkane data show evidence of fossil fuel input and no evidence for a terrestrial plant source fraction. Barger and Garrett^{1,3} analysed aerosol samples from the eastern equatorial and North central Pacific for fatty acids in the C_{12} – C_{20} range. As no data are reported for compounds > C_{20} the fatty acid land plant component of these samples cannot be assessed.

Our preliminary data therefore show the presence of high molecular weight lipid class compounds in marine aerosols at a remote open ocean site.

The individual compound distribution data strongly indicate a continental source, probably epicuticular vascular plant waxes, for a significant portion of this material. The temporal variation, a decreasing trend in aluminosilicates, ^{210}Pb and total lipids with time, strongly suggest that similar transport mechanisms and source functions may be involved. Further verification of these interpretations requires size-fractionated sampling (by cascade impactor), continued maintenance of ultra-clean sampling conditions, associated meteorological controls and other chemical analyses to infer sources and transport processes from lipid concentration data.

We thank the Oceanography Section of the NSF for support (OCE 77-12914) as part of the SEAREX Program and R. A. Duce, C. Lee and J. Volkman for critical comments. We also thank J. Alford, E. Atlas, R. Cayer, C. Snipes, the staff of the University of Hawaii's Mid-Pacific Marine Laboratory and the Department of Energy for support during the field work in Enewetak.

Received 7 January; accepted 9 March 1981.

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Floristic changes indicate a cooling climate in the Eocene of southern England

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A major climatic cooling at the close of the Eocene (the 'terminal Eocene event') is suggested by studies of leaf floras in North America^{1,2}; and by oxygen isotope studies, of mollusc shells from the North Sea³ and of planktonic and benthic foraminifera from the North and South Pacific⁴. Climatic fluctuations in the French Palaeogene⁵⁻⁸ inferred from palynological studies, are difficult to interpret in terms of this single event. The Pacific faunal studies⁹ and preliminary palynological evidence from the Isle of Wight, southern England⁹ indicates an onset of cooling earlier in the Eocene. We have therefore examined fossil floras (fruits, seeds, pollen and spores) from the Palaeogene deposits of the London and Hampshire Basins of southern England which form a continuous sequence from the early Eocene. We find no evidence for a sudden climatic change at the end of the Eocene (taken to be near the base of the Bembridge Marls in the Hampshire Basin¹⁰). Our evidence, in the form of two major periods of floristic change, suggests a more gradual cooling, commencing in the latest early Eocene.

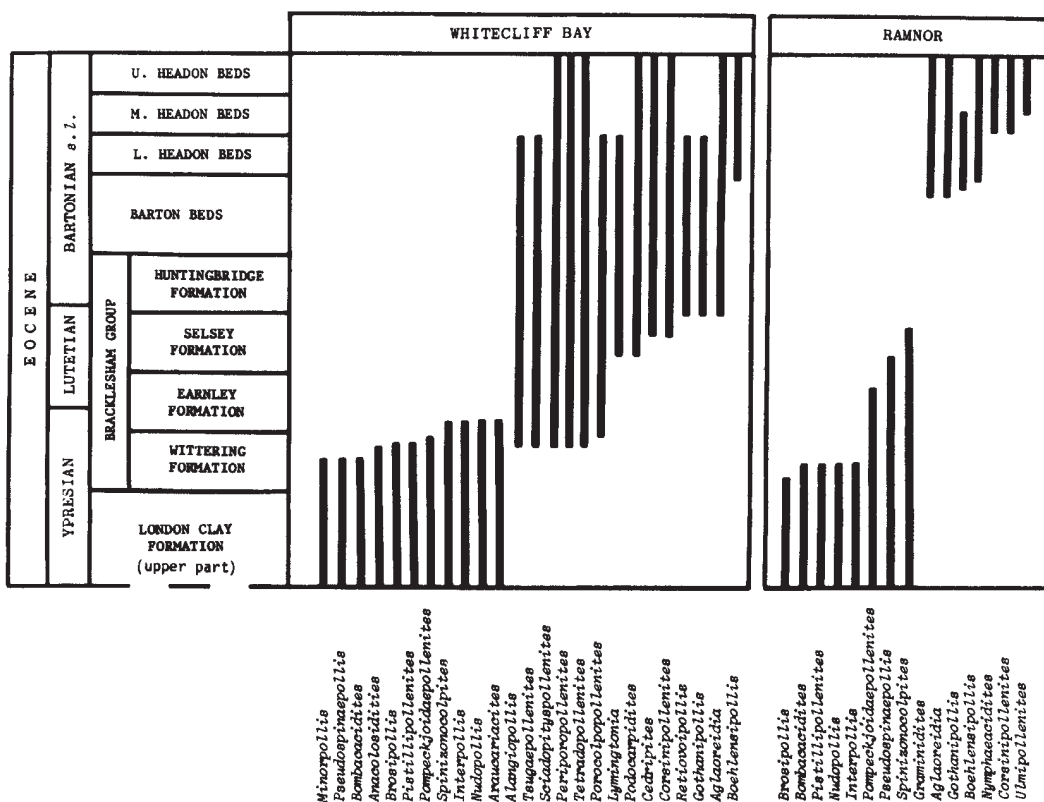
The evidence of floristic change during the Eocene has resulted from recent work on fossil pollen and spores from coastal sections (Hampshire Basin⁹) and Institute of Geological Sciences boreholes (Hampshire Basin¹¹) together with fossil angiosperm fruits and seeds (throughout the area¹²). Palyno-

logical evidence is available as percentage frequency counts from some 200 samples representing two Eocene sections in the Hampshire Basin. The sections were at Whitecliff Bay, Isle of Wight (SZ 643 864) which includes the type section of the Bracklesham Beds¹³, and the I.G.S. Ramnor Inclosure borehole near Brockenhurst (SU 3114 0475). Of more than 100 form-genera present within the assemblages, many occur throughout the Hampshire Basin Tertiary and are known to range throughout the Eocene of North-west Europe. Of particular significance are those form-genera with a restricted stratigraphical range within the sections studied (Fig. 1). In the Whitecliff Bay section, 13 form-genera first appear within the Bracklesham Group and one other in the Lower Headon Beds; 11 extinctions occur in the lower part of the Bracklesham Group and another six at the end of the Lower Headon Beds. In the Ramnor borehole, seven form-genera first appear within the Barton and Headon Beds and eight become extinct within the Bracklesham Group.

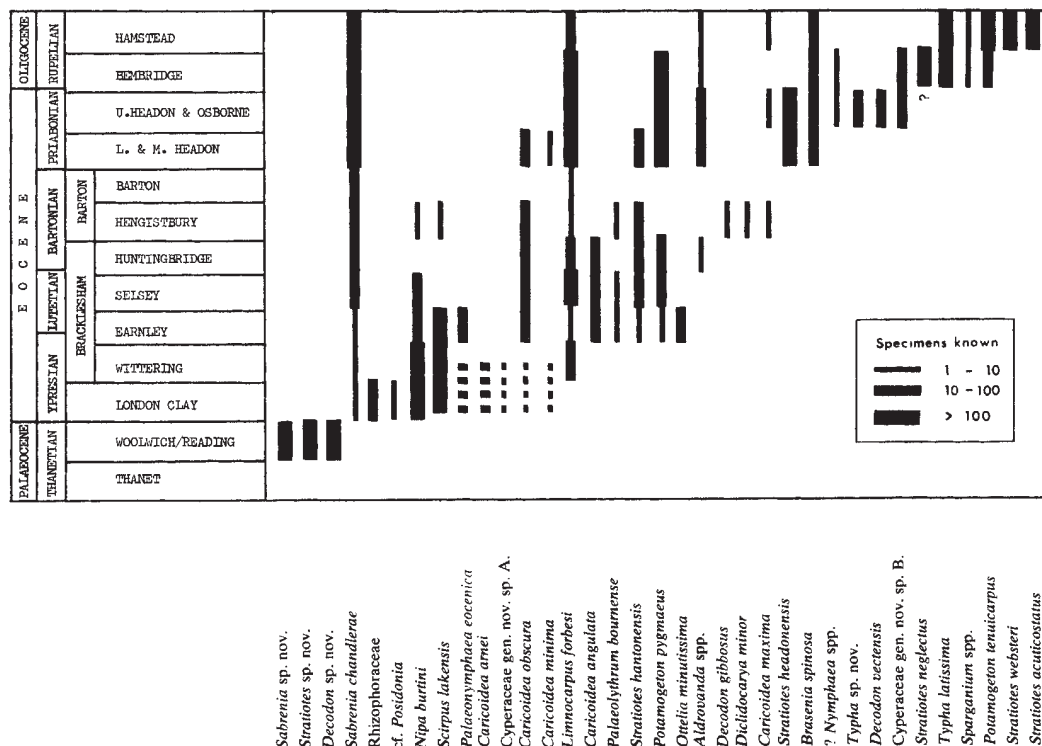
Among the fossil fruits and seeds, 72 angiosperm taxa have a stratigraphical range within the Palaeogene of southern England (data from the same sources as those on Fig. 2). Of these taxa, 45% first appear within the London Clay and 13% within the Bracklesham Group. Major extinctions occur within the Bracklesham Group (14%) and the Lower Headon Beds (39%).

The sedimentary sequence which we are studying was deposited in a marginal area affected by marine transgressions and regressions. It is recognized that facies variation in such a depositional environment could account for the apparent macrofloristic changes. Therefore, detailed analyses are presented for 35 taxa (including 22 already considered above) all of which have their nearest living relatives occupying a fresh or brackish, aquatic or marginal aquatic habitat (Fig. 2). Lithofacies representing deposition in these habitats occur extensively throughout the study area, whereas those containing land-derived macrofossils have a more sporadic occurrence. Macrofloristic data in Fig. 2 are considered to represent a close approximation to the true distribution of the plants which bore these fossil fruits and seeds. This is further supported by evidence that many of the fossil occurrences cross facies boundaries within the sequence. The macro- and microfloral changes which

Fig. 1 Stratigraphical ranges of selected pollen and spore form-genera in the Eocene of the Whitecliff Bay coastal section and Ramnor Inclosure borehole. Stratigraphy follows that of Curry *et al.*¹⁰ and European stage names extrapolated from refs 10, 19.



In the upper Bracklesham of Whitecliff Bay a second sequence of microfloristic changes begins. It is marked by the introduction of new form-genera characteristic of the overlying Barton and Headon Beds, together with an increased abundance of spores, bisaccate and inaperturate pollen (representing ferns and conifers). This fern-conifer association is a dominant feature of palynological assemblages in the overlying Palaeogene sediments of the Hampshire Basin. Further changes occur



localities of Dorset Pipe Clays and Bagshot Beds^{12,25} have been placed within the London Clay and Wittering. Short dashed lines indicate uncertainty within this range. The positions of other plant bearing strata may be extrapolated from ref. 10.

These results indicate two main periods of floristic change during the Eocene in Britain. The first originated in the Wittering and continued during the Lutetian. The second originated in the uppermost Selsey and is expressed mainly within the Barton and Headon Beds. This second change culminates in the development of extensive reed swamp floodplain vegetation^{12,17}, the extinction of many tropical and subtropical floral elements and the dominance of pteridophyte spores and conifer pollen in the microfloral assemblages. Both of these floristic changes may indicate a progressive lowering of land surface temperature during the Middle Eocene in southern

England, the second possibly more rapid than the first. The cooling which we suggest these changes may imply took place over a period of 15 Myr. This is of interest in relation to the palaeotemperature curves for the North Sea^{3,18}, which indicate a sudden and rapid cooling at the close of the Eocene. The floristic changes shown in our results from the Palaeogene of southern England suggest that climatic events were more complex than is indicated by the recognition of a single 'terminal Eocene event'.

M.E.C. acknowledges receipt of a NERC research fellowship during the tenure of which part of this work was completed. Results from the Ramnor borehole are published with permission of the director of Institute of Geological Sciences.

Received 7 January; accepted 10 March 1980.

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A new assessment of *Rhyniella*, the earliest known insect, from the Devonian of Rhynie, Scotland

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The search for insects (Class Hexapoda) in rocks of Devonian and Lower Carboniferous age (325–415 Myr BP) has continued for many years because so little is known of the early history of these diverse and abundant animals. In 1926, four head capsules of an insect, named *Rhyniella praecursor*, were discovered in Lower Devonian chert from Rhynie, Aberdeenshire¹ by examining thin fragments of this chert, which are rather translucent, like amber. Subsequently, about 10 additional specimens were found showing the legs and thorax of this insect². It has become generally accepted that *R. praecursor* belongs to the living Order Collembola or springtails, a widespread, flightless group of insects. However, several characters of Collembola were not preserved in the fossils and doubts remained. Furthermore, the apparent similarity of *Rhyniella* to some specialized modern Collembola³ has suggested that *Rhyniella* was a modern contaminant⁴. Our research shows that *Rhyniella* is not a contaminant; we have also found the first intact abdomen of *Rhyniella* which has a special locomotory appendage similar to that of modern Collembola.

We initially decided to search the Rhynie Chert because it is the only formation before the Upper Carboniferous to have yielded insect remains. The samples we examined were excavated during the International Botanical Congress in 1964 by J. Pettitt and C. Shute of the Department of Palaeontology,

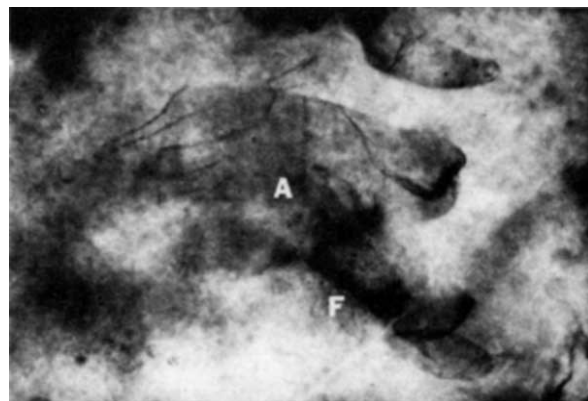


Fig. 1 Photograph of *R. praecursor*, fossilized in chert from Rhynie. A, abdomen; F, furcula.

British Museum (Natural History). However, no insects were found although several arachnids were discovered. We then re-examined the original material collected over 50 yr ago; in a few cases the chert was thick and thus was ground and repolished to see the contents more clearly. As a result, a new specimen was revealed which consists of a thorax with legs and the first-discovered complete abdomen. Extending from the ventral surface of the abdomen is a structure corresponding to the furcula ('spring') of Recent Collembola (Figs 1, 2). Only the hind end of the *Rhyniella* abdomen can be seen in Fig. 1 because the body is oblique to the plane of view. The presence of a furcula confirms that *Rhyniella* was a springtail; the total length of the insect was ~1.5 mm. Figure 2 shows a Recent springtail which has a slender apex (dentes) to the furcula.



Fig. 2 A Recent springtail. A, abdomen; F, furcula.

Examination of several specimens of *Rhyniella* showed that they were completely fossilized in chert and did not look like modern contaminants. They were examined petrologically in polarized light by A. R. Woolley and the enclosing chert was found to be homogeneous and certainly represented a single phase of mineral growth. One *Rhyniella* specimen was associated with a plant fragment in homogeneous chert and J. B. Richardson confirmed that the petrification of this was identical to other Rhynie plants of undoubtedly Devonian age. We conclude that *Rhyniella praecursor* is of Lower Devonian age (possibly Siegenian).

Thus Collembola were established and already developing their specialized mode of locomotion some 380 Myr ago. The relationship of *Rhyniella* with Recent Collembola Neanuridae is doubtful and a fuller account will be published elsewhere.

We thank P. N. Lawrence for advice on Collembola.

Received 24 November 1980; accepted 30 March 1981.

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The role of GABAergic inhibition in the cortical effects of monocular deprivation

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In cats and monkeys, monocular deprivation in early life results in an extensive reduction of the capability of the deprived eye to activate visual cortical cells¹, apparently because of changes taking place in the visual cortex itself rather than at an earlier stage in the visual pathway². The nature of the change taking place in the cortex is the subject of some controversy. One line of evidence favours a redistribution of the excitatory terminals relating to the two eye inputs³⁻⁵ whilst another suggests that the deprived eye input is in some way suppressed⁶, possibly by γ -aminobutyric acid (GABA)-mediated intracortical inhibition⁷. We have now sought to clarify the role of this type of inhibitory influence in monocularly deprived cats, by ascertaining whether there is an enhancement of the deprived eye input to visual cortical cells during the iontophoretic application of the GABA antagonist, *N*-methyl-bicuculline (NMB). Our experiments show that although intracortical inhibition may submerge the deprived eye input to some cells, it is unlikely to precipitate the cortical effects of monocular deprivation. The redistribution of excitatory terminals would seem to be a more significant factor.

Six cats were used for the experiments—three early monocularly deprived animals, with lid suture at the time of natural eye opening and three late monocularly deprived animals, with lid suture carried out 2 weeks after eye opening. All recordings were made when the animals were in the age range 12–24 months. After initial surgical preparation the animals were paralysed with Flaxedil and maintained on an anaesthetic mixture of 75% N₂O–25% O₂ and 0.1–0.4% Halothane. Full details of experimental procedures are given elsewhere⁸. Recordings were made extracellularly from the central barrel of five-barrel micropipettes, the other barrels containing respectively NMB (two barrels), DL-homocysteate and GABA. Cell properties were documented on line by construction of peristimulus time histograms (PSTHs) of the responses to a slit of light moving over the receptive field in the normal eye and the corresponding location in the deprived eye. Ocular dominance was assessed before, during and after NMB application. Retinal landmarks were periodically back-projected onto the stimulus projection screen, to check the relative position of the two eyes. Great care was taken in varying stimulus location and orientation in the deprived eye during NMB application to ensure that errors in estimation of corresponding field location and eye rotation did not cause weak response fields to be missed.

Tests were completed on 51 cells, all initially exclusively dominated by the normal eye. Of these cells, 29% (15) developed a response, though sometimes fairly weak, to stimulation of the deprived eye during NMB application. The results for the early and late monocularly deprived animals were essentially the same, with respectively 28% (6/21) and 30% (9/30) of the cells studied in each group developing deprived eye inputs. The pooled results for early and late monocularly deprived animals are summarized in Fig. 1. These should be compared

with similar data obtained for monocularly dominated cells in normal cats⁹, where ~50% of the cells showed a change in ocular dominance during NMB application. This is a significantly larger percentage than that seen in the present sample of cells and suggests that intracortical inhibition exerts a more powerful influence on ocular dominance in the cortex of normal as opposed to monocularly deprived cats.

An example of the action of NMB on the ocular dominance of a complex cell is illustrated in Fig. 2. This shows the effect of two

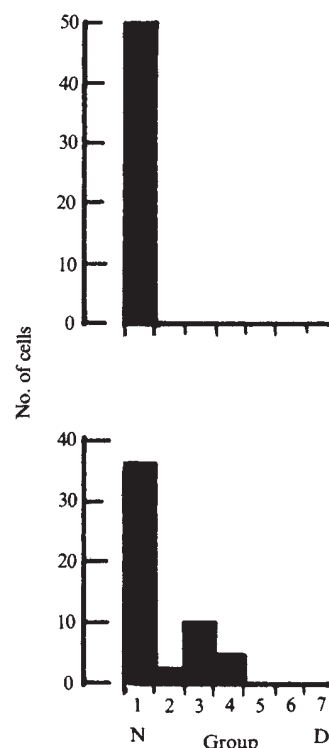


Fig. 1 Histograms showing the effect of iontophoretic application of NMB on the ocular dominance of cells in the visual cortex of six monocularly deprived cats. The ocular dominance of the cells has been classified on the seven-point scale described by Hubel and Wiesel¹⁵. Group '1' cells are exclusively driven by the contralateral eye, group 7 by the ipsilateral eye, while those in group 4 are equally driven by both eyes. Cells in groups 5 and 6, and 3 and 2, are slightly and strongly dominated by ipsilateral and contralateral eyes, respectively. Recordings were made in area 17 both ipsilateral and contralateral to the deprived eye. All the cells studied were initially exclusively dominated by the normal eye and hence fell into group 1 or 7. In the histograms, the results from ipsilateral area 17 have been pooled with those from contralateral area 17 so that group 1 represents the normal eye (N) whilst group 7 represents the deprived eye (D). A shift in ocular dominance from left to right represents a change in favour of the deprived eye. The upper histogram shows the control ocular dominance distribution and the lower histogram the distribution during NMB application. The largest shift in ocular dominance was to group 4 with equal driving by normal and deprived eye.

successive periods of NMB application on the response to movement of an optimally oriented slit of light over the normal eye's field and the corresponding location in the deprived eye. During each period of NMB application there was a loss of directional selectivity and an increase in magnitude of the normal eye response, together with the appearance of a smaller but clear response in the deprived eye. These changes were followed each time by recovery of the original properties after termination of the application. The results for all the cells developing a deprived eye input were reversible and

reproducible. We also observed that the response characteristics obtained from stimulation of the normal and deprived eyes were often quite distinct. However, we are not in a position to draw any conclusions regarding possible differences in the overall response specificity exhibited by the latent deprived eye receptive fields and the normal eye fields⁶. The problem is that many of the receptive field properties of visual cortical cells, including orientation selectivity, are partially or entirely dependent on the action of GABA-mediated intracortical inhibition^{8,10-12}. Consequently, apart from any action on ocular dominance, iontophoretic application of NMB also influences those processes generating or enhancing receptive field specificity. Thus, we cannot say whether a lack of specificity in the deprived eye input reflects the action of NMB on GABA-mediated inhibitory mechanisms that generate the specificity, or the absence of such mechanisms operating with respect to the deprived eye input. The report⁷ that intravenous injection of bicuculline revealed deprived eye fields with normal properties in monocularly deprived cats is puzzling and would seem to indicate that the intravenous bicuculline was having little effect on intracortical GABAergic synapses.

One of the side effects of NMB application is a marked elevation in resting discharge level. This may be partly due to release of the cell from tonic GABAergic inhibitory effects and partly to a direct excitatory action of the NMB. The lower record in Fig. 2 shows an attempt to control for the possible effects of the elevated resting discharge by generating an increased discharge level by the iontophoretic application of an excitatory amino acid, DL-homocysteate. In these circumstances, at a similar resting discharge level to that seen with NMB, there is no sign of a visual response from the deprived eye. This suggests that in these experiments it is the block of GABAergic inhibition and not the increase in resting discharge as such that reveals the deprived eye input. Indeed, it seems that, for many visual cortical cells, an increase in resting discharge in the absence of a block of GABAergic inhibition decreases responsiveness to visual stimuli and increases the effectiveness of visually evoked inhibitory influences, possibly via recurrent collateral feedback⁸. It is interesting that Blakemore, Hawken and Mark (in preparation) have shown that iontophoretic application of DL-homocysteate at currents sufficient to depolarize cells without causing considerable spontaneous discharge will reveal an input from the deprived eye to many neurones in briefly monocularly deprived kittens.

In experiments of this type negative results must be interpreted with greater caution than positive ones. The fact that 70% of the cells failed to show an input from the deprived eye during NMB application suggests that intracortical GABA-mediated inhibition is making no significant contribution to their domination by the normal eye. However, it could reflect a lack of pharmacological effectiveness of the NMB or an inadequate concentration of the drug at the actual inhibitory synapses involved. Control observations carried out on all cells included in the present sample showed that these possibilities were not significantly influencing the results. We determined the ejecting current necessary for iontophoretically applied GABA to suppress completely the cell's response to motion of an optimal stimulus over the normal eye receptive field. Data taken during NMB application were only considered acceptable when the previously determined suppressive dose of GABA failed to suppress the cells driven activity. This is illustrated in Fig. 3 for a complex cell that retained its ocular dominance during NMB application. The PSTHs show responses respectively to normal eye stimulation, deprived eye stimulation and then normal eye stimulation again but in the presence of iontophoretic application of GABA. During NMB application, although there is no response from the deprived eye, the application of GABA fails to suppress the normal eye response, indicating that the NMB is pharmacologically effective. With this type of pharmacological check and other functional criteria, we assessed the potency of NMB at the synaptic level. For all cells, NMB application resulted in a reduction or loss of inhibition-dependent receptive

field properties^{8,10-12} such as direction and orientation selectivity, together with an increased magnitude of response suggesting a loss of tonic inhibitory influences. These observations indicate that neither variations in the pharmacological potency of NMB nor its functional effectiveness at inhibitory synapses were significant factors contributing to the failure to reveal deprived eye receptive field properties in 70% of the cells studied. However, inhibitory influences other than those mediated by GABA could be suppressing the deprived eye input to some cells. Although strong evidence supports the role of

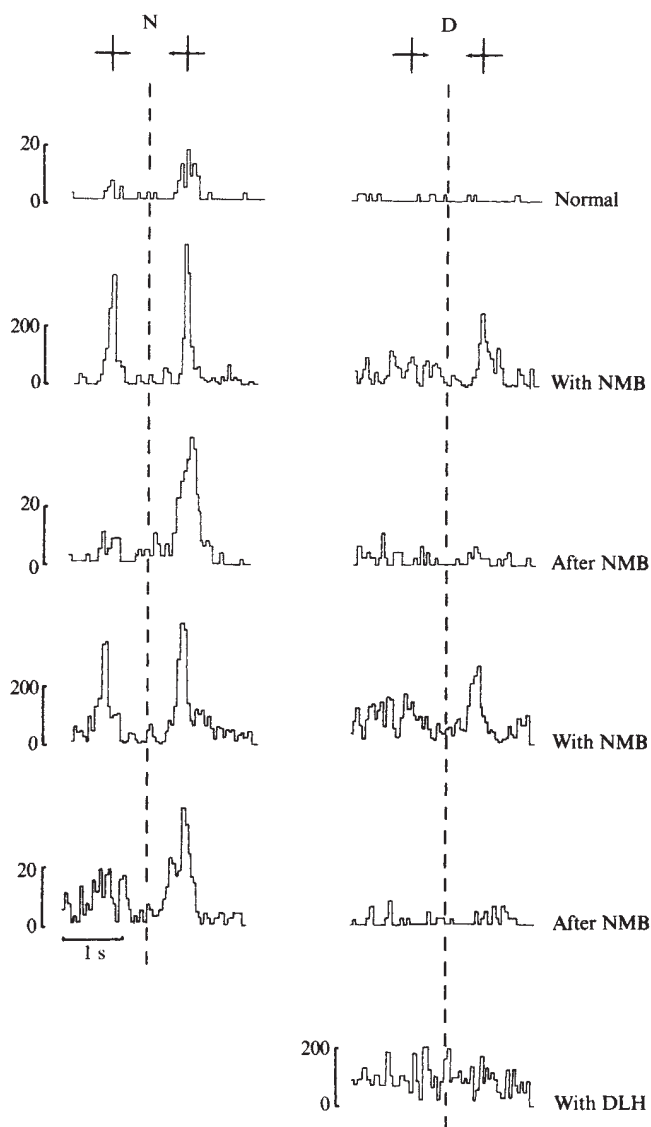


Fig. 2 Responses of a complex cell showing a shift in ocular dominance during iontophoretic application of NMB. Each PSTH shows the responses to 25 sweeps of an optimally oriented slit of light moving forwards (to left of dotted line) and backwards (to right of dotted line) over the receptive field in the normal eye (N) and the corresponding location in the deprived eye (D). Bin size 50 ms. Vertical calibration indicates the number of spikes per bin accumulated in 25 trials. PSTHs document the effect of and recovery from two successive periods of the iontophoretic application of NMB (ejecting current 60 nA). The bottom right PSTH shows that increasing the resting discharge by iontophoretically applying an excitatory amino acid, DL-homocysteate (DLH, ejecting current 8 nA), does not restore a response in the deprived eye.

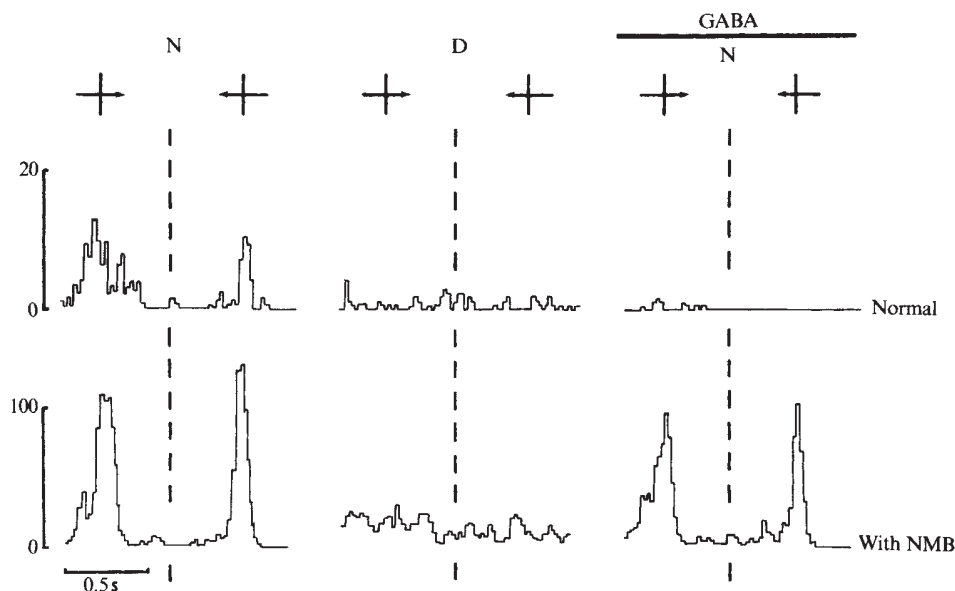


Fig. 3 Responses of complex cell showing pharmacological block of GABA action during NMB application but no deprived eye input. PSTHs show response to an optimally oriented slit moving over the normal eye receptive field, corresponding location in the deprived eye and normal eye receptive field again but during iontophoretic application of GABA (15 nA, marked by bar over records). Details of PSTHs as for Fig. 2. Upper records show response before and lower records response during NMB application (ejecting current 60 nA). Note that GABA blocks normal eye-driven response before, but not during, NMB application.

GABA as a major inhibitory transmitter in the visual cortex^{10,13,14}, other inhibitory transmitters could be involved in this location. It is therefore important to emphasize that the present data only refer to GABA-mediated inhibition.

We conclude that in monocularly deprived animals 30% of the cells exclusively dominated by the normal eye actually receive a deprived eye input that is normally submerged, at least in part, as a result of the action of local GABAergic inhibitory influences acting on the cells. It is not clear whether plastic changes in the GABAergic inhibitory processes *per se* are an important or even significant factor contributing to the actual shift in ocular dominance seen during monocular deprivation. Note that none of the cells studied was ever dominated by the deprived eye during NMB application. If intracortical inhibitory mechanisms in normal animals enhance the contrast between adjacent ocular dominance columns⁹, these same processes initiated from the greatly extended normal eye ocular dominance columns could suppress the output from the small islands of deprived eye input known to exist in layer IV^{3,6}. Thus the precipitating factor would be the redistribution of excitatory terminals from the two eyes and not the specific action of intracortical inhibitory processes. Some of our results may also reflect a very simple nonspecific increase in responsiveness due to the removal of the tonic GABAergic inhibitory influences, whereby a small deprived eye input becomes apparent because the cell is more responsive to its input. These nonspecific effects would not, however, apply to the few cells that became equally driven by both eyes. We postulate a passive, rather than an active, role for intracortical GABAergic inhibition in the effects of monocular deprivation.

Support from the MRC and Wellcome Trust is gratefully acknowledged.

Morphine and δ -opiate agonists locally stimulate *in vivo* dopamine release in cat caudate nucleus

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The peripheral administration of morphine¹⁻⁴ or the intraventricular injection of either Met-enkephalin or D-Ala²-Met-enkephalinamide⁴ (a synthetic enkephalin analogue resistant to enzyme degradation) enhanced dopamine (DA) turnover in the rat striatum. These effects, in turn, could be blocked by the opiate antagonist naloxone¹⁻⁴. As the striatum contains a high density of opiate receptors⁵, these drugs may be affecting DA metabolism by a local action. This hypothesis is supported by the evidence that morphine still enhanced striatal DA turnover shortly after transection of the nigro-striatal DA pathway⁶. Furthermore, D-Ala²-Met-enkephalinamide directly injected into the striatum significantly increased striatal DA turnover⁴. However, the mechanism by which opiates stimulate DA turnover is not clear because they have not yet been shown to enhance DA outflow in *in vitro* release studies⁷⁻⁹. We have therefore now examined *in vivo* the local effects of opiates on the release of DA in the cat caudate nucleus. We find that both morphine and more markedly, D-Ala²-Met-enkephalinamide stimulate DA release. The action of morphine is antagonized by naloxone, but that of the synthetic enkephalin is not. Because the effects of D-Ala²-Met-enkephalinamide are similar to those of the peripheral δ -receptor agonist Tyr-D-Ser-Gly-Phe-Leu-Thr, we conclude that the synthetic enkephalin acts on δ -receptors.

Experiments were carried out on adult cats of both sexes lightly anaesthetized with halothane, implanted with a push-pull cannula in one caudate nucleus to measure continuously the release of ³H-DA synthesized from ³H-tyrosine as previously described¹⁰. In these conditions, the spontaneous release of ³H-DA is dependent on nerve activity and can be influenced by the local application of various transmitters¹¹. The technique consists in the delivery (1.8 ml h⁻¹) of an artificial cerebrospinal fluid containing ³H-tyrosine (50 Ci mmol⁻¹, 50 μ Ci ml⁻¹) to the push-pull cannula and in the estimation of ³H-DA in serial

Received 3 November 1980; accepted 9 March 1981.

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10-min superfusate fractions. ^3H -DA is separated from ^3H -tyrosine and ^3H -labelled metabolites by ion-exchange chromatography and alumina adsorption¹². The drugs tested were added for 30 min in the superfusing fluid 160 min after the beginning of the superfusion with ^3H -tyrosine, that is, at least 1 h after the steady-state level of the spontaneous ^3H -DA release had been reached. As the effect of a single application of a compound can only be estimated in one experiment with this preparation and several experiments are required for statistical analysis, only one concentration of each compound was used in most cases.

Morphine (10^{-6} M) significantly stimulated the local release of ^3H -DA (Fig. 1a). This effect was seen during the last 20 min of the drug application and persisted in the two following fractions after its removal from the superfusing fluid. Naloxone (10^{-6} M) simultaneously added with morphine (10^{-6} M) to the superfusing fluid completely prevented the stimulatory effect of morphine on ^3H -DA release (Fig. 1b). In fact, the pattern of ^3H -DA release seen in the presence of both drugs was similar to that observed with naloxone (10^{-6} M) alone, which induced a delayed reduction in ^3H -DA release (Fig. 1c).

D-Ala²-Met-enkephalinamide, used at a concentration (10^{-6} M) similar to that of morphine also stimulated ^3H -DA release (Fig. 2a), but in this case, the stimulatory effect was immediate, more pronounced and biphasic. The initial increase in ^3H -DA release lasted only 10 min whereas the second increase in ^3H -transmitter release was seen as soon as the enkephalin analogue was removed from the superfusing medium. In contrast to the results observed with morphine, naloxone (10^{-6} M) did not significantly modify the biphasic stimulatory effect of D-Ala²-Met-enkephalinamide (10^{-6} M) when simultaneously added with the agonist (Fig. 2b). It was still ineffective at a concentration of 10^{-5} M (data not shown). Higher concentrations of naloxone were not used because they have been shown to exert non-specific effects^{13,14}.

Pharmacological studies on peripheral tissues have revealed the presence of various types of opiate receptor¹⁵. Furthermore, recent binding studies with striatal membranes using ^3H -

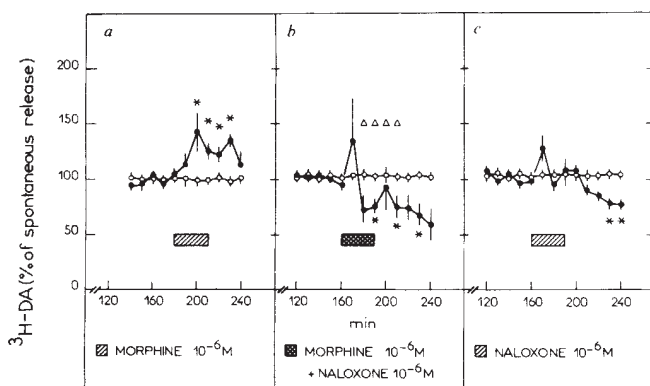


Fig. 1 Effects of morphine (a), combined morphine and naloxone (b) or naloxone (c) applications into the cat caudate nucleus on the release of ^3H -dopamine (^3H -DA). A push-pull cannula was implanted in the caudate nucleus in halothane-anaesthetized cats. This structure was superfused with an artificial cerebrospinal fluid containing L-[3,5- ^3H]tyrosine (50 Ci mmol⁻¹, 50 $\mu\text{Ci ml}^{-1}$, 1.8 ml h⁻¹). ^3H -DA was estimated in 10-min successive superfusate fractions. An average of 0.6 nCi of ^3H -DA was released in superfusate fractions (30 times the blank value). Morphine (10^{-6} M), naloxone (10^{-6} M) or both morphine (10^{-6} M) and naloxone (10^{-6} M) were introduced for 30 min into the superfusing fluid (hatched bars). In each animal, ^3H -DA in each successive fraction was expressed as a percentage of an average spontaneous release calculated from the five fractions collected before the treatment. Data are the mean $\pm$ s.e.m. of results obtained with 6(a), 5 (b) or 14 (c) animals (●). * $P < 0.05$ when compared with corresponding control values obtained in six untreated animals (○). $\Delta P < 0.05$ when compared with values obtained in the presence of morphine (10^{-6} M) alone.

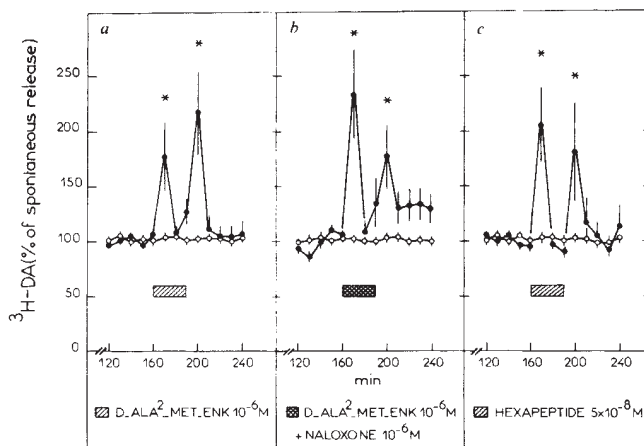


Fig. 2 Effects of D-Ala²-Met-enkephalinamide (D-Ala²-Met-Enk) in the absence (a) or presence (b) of naloxone and of Tyr-D-Ser-Gly-Phe-Leu-Thr (c). Seven (a), eight (b) or nine (c) animals were treated as described in Fig. 1 legend except that D-Ala²-Met-enkephalinamide (10^{-6} M) or D-Ala²-Met-enkephalinamide (10^{-6} M) and naloxone (10^{-6} M) or the hexapeptide (5×10^{-8} M) were applied to the caudate nucleus for 30 min (hatched bars). Data are calculated and expressed as in Fig. 1. * $P < 0.05$ when compared with control values obtained in six untreated cats (○).

morphine and ^3H -D-Ala²-D-Leu⁵-enkephalin as ligands have revealed a twofold higher amount of δ versus μ opiate receptors in the rat striatum¹⁶. As morphine seems less effective than the enkephalin derivative in stimulating the *in vivo* release of ^3H -DA and as the effect of D-Ala²-Met-enkephalinamide was not antagonized by an equimolar concentration of naloxone, the effects of this derivative in our experiments could be mainly mediated by δ -receptors. Indeed, although naloxone can block various peripheral opiate receptors, higher concentrations of this antagonist are required to block δ - than μ -receptors¹⁵. Moreover, naloxone was shown to be 20 times less potent on δ - than on μ -receptors in binding studies with rat striatal membranes¹⁶.

Gacel and his collaborators have recently synthesized an hexapeptide, Tyr-D-Ser-Gly-Phe-Leu-Thr, which is 620 times more potent on mouse vas deferens δ -receptors than on guinea pig ileum μ -receptors¹⁷, and so we tested this compound in our preparation. At a concentration (5×10^{-8} M) 100 times lower than the ED₅₀ value for its action on peripheral μ -receptors, Tyr-D-Ser-Gly-Phe-Leu-Thr had a marked biphasic stimulatory effect on ^3H -DA release similar to that observed with D-Ala²-Met-enkephalinamide (10^{-6} M) (Fig. 2c), further suggesting a δ -receptor-mediated action of D-Ala²-Met-enkephalinamide on ^3H -DA release.

These experiments demonstrate for the first time that opiates stimulate the *in vivo* release of DA in the caudate nucleus, and support previous proposals that morphine or enkephalin derivatives modulate DA metabolism by a local action in the striatum⁴. In fact, as morphine, D-Ala²-Met-enkephalinamide and the agonist Tyr-D-Ser-Gly-Phe-Leu-Thr were directly applied into the caudate nucleus in our experiments, the opiate receptors involved in the effects observed must be located within this structure. A stimulation of the *in vivo* spontaneous release of ^3H -DA in the cat caudate nucleus has also been seen during the local application of acetylcholine (10^{-5} M)¹⁸ or γ -amino-butyric acid (10^{-5} M)¹⁹, which induced a biphasic stimulatory effect similar to that observed with D-Ala²-Met-enkephalinamide and Tyr-D-Ser-Gly-Phe-Leu-Thr. The rapid interruption of the initial stimulatory effect seen in all these cases could be attributed to the action of the released DA on DA autoreceptors²⁰.

Combined lesion and binding studies in the rat have revealed that opiate receptors are heterogeneously distributed within the striatum. Some are located on DA terminals⁵ while others are

present on striatal cell bodies or dendrites²¹. Furthermore, as opiate binding sites are still detectable after the destruction of the nigrostriatal DA neurones by 6-hydroxydopamine and the kainic acid-induced degeneration of striatal perikarya, these remaining binding sites could be located on striatal afferent fibres other than the DA terminals²¹. Opiate receptors located on striatal cell bodies and dendrites do not seem to be essential for the regulation of DA metabolism because opiates enhance DA turnover after kainic acid-induced degeneration of the striatal perikarya⁴. The opiate receptors located on DA terminals or on other afferent fibres could be involved in the changes in DA release observed in our experiments.

In vitro studies have shown that several transmitters exert a direct or indirect presynaptic influence on DA release¹¹. In preliminary experiments, we examined the effects of opiates on the spontaneous release of ³H-DA synthesized from ³H-tyrosine in rat striatal slices and found that morphine (10^{-6} M, 5×10^{-6} M) was ineffective whereas D-Ala²-Met-enkephalinamide (10^{-6} M) significantly increased ³H-DA release. This further suggests that morphine and D-Ala²-Met-enkephalinamide by different processes.

Opiates have been shown to modulate the *in vitro* release of acetylcholine in the myenteric plexus²², substance P in the trigeminal nuclei²³ and noradrenaline in the cerebral cortex⁸. In all these studies the effects of opiates were antagonized by naloxone, as was the case in our *in vivo* experiments with the morphine-induced release of ³H-DA. Further studies will be required using pure μ -receptor agonists to determine whether or not the action of morphine is mediated by μ -receptors. In contrast, naloxone (10^{-6} M and 10^{-5} M) did not prevent the effect of D-Ala²-Met-enkephalinamide (10^{-6} M) on ³H-DA release, suggesting that μ opiate receptors were not involved.

Biochemical and immunohistochemical studies have demonstrated a rich innervation of the striatum by enkephalinergic neurones^{24,25}, and experiments in animals implanted with push-pull cannulae have shown that Met-enkephalin is spontaneously released *in vivo* from the cat caudate nucleus (3.5 pg per fraction per 0.5 ml)²⁵. It thus remains to be established if endogenous opiates regulate the release of DA from DA terminals. The availability of a specific and potent antagonist of opiate receptors of the δ -type will be required to determine the precise contribution of enkephalinergic neurones in the presynaptic regulation of DA release in the caudate nucleus.

D-Ala²-Met-enkephalinamide was provided by Dr Y. Audi-gier (Toulouse) and the δ -agonist hexapeptide by Dr Roques (Paris). We thank them for their help and fruitful discussions and Mr M. Desban for assistance. Naloxone was given by Endo Laboratories. This study was supported by grants from INSERM (ATP 58.78.90), DRET (78.004), and Rhône-Poulenc SA. T. D. Reisine is a NINCDS fellow (1 F32 NS 0 6467.01) and A. C. is a research fellow of Rhône-Poulenc SA.

Received 22 September 1980; accepted 9 March 1981.

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Heavy meromyosin cross-links thin filaments in striated muscle myofibrils

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Studies of the interaction of proteolytic fragments of myosin with actin filaments in solution have indicated that the two heads of the myosin molecule¹ can bind to adjacent subunits in the same actin filament^{2,3}. However, geometrical considerations led Offer and Elliott⁴ to the conclusion that in the highly ordered filament lattice present in insect flight muscle, tighter binding would occur if the two heads bound to different actin filaments. Indeed, rigidity measurements⁵, electron microscope observations⁶ and superprecipitation experiments⁷ support the idea that heavy meromyosin (HMM), the double-headed myosin fragment, can cross-link actin filaments in solution. We now provide an experimental demonstration of the cross-linking of thin filaments in muscle by double-headed myosin species. In striated muscle, the thin filament lattice can be stabilized by either the Z-bands or complex formation with myosin. Removal of the Z-bands by a proteolytic enzyme⁸ or extraction of myosin, leaves the actin-containing I-bands intact. However, if both Z-bands and myosin are removed, the I-bands should disintegrate into single, dispersed, filaments. We found that this could be prevented by preincubation with HMM, but the single-headed HMM subfragment-1 (HMM S-1) was totally ineffective. This strongly suggests that the stabilization by HMM of the filamentous lattice is a result of inter-filament cross-linking.

Myofibrils were prepared from glycerinated rabbit psoas muscle, homogenized in the Omni-Mixer (see Fig. 1 legend for details). HMM and HMM S-1 were prepared by the chymotryptic digestion of myosin described elsewhere⁹ and HMM was further purified by fractional precipitation with ammonium sulphate. Myosin contamination of the fragments was assessed by SDS-polyacrylamide gel electrophoresis; the HMM S-1 preparations had no detectable contamination and that in HMM was estimated to be <1% (by weight).

Figure 1 shows a series of manipulations of a single myofibril in rigor solution. An intact myofibril (Fig. 1a) was treated with the myosin-extracting solution, resulting in a myosin-free ghost (Fig. 1b) in which the Z-lines are clearly visible. If such ghosts are then treated with a trypsin solution, they disintegrate within a few seconds (not shown). The ghosts were first incubated with HMM, which when bound to the thin filaments enhanced the phase image of the ghost, but the Z-lines were still clearly visible (Fig. 1c). After treatment with trypsin-containing solution, the Z-lines were digested and disappeared completely in 60 s (Fig. 1d). Nevertheless, as clearly demonstrated in Fig. 1d, and reproduced in 12 identical experiments with 3 different HMM preparations (including HMM containing 'short' S-2), the ghost remained intact. If an identical procedure is followed with HMM S-1 instead of HMM, the ghosts disappear within a few seconds after the application of the Z-line-digesting solution (not shown).

We considered the possibility that residual myosin in the HMM preparations (at most 1% by weight, see above) was responsible for this effect. When the myofibrillar ghosts were pre-incubated with a rigor solution containing 1.5 mg ml^{-1} HMM S-1 and 0.15 mg ml^{-1} myosin, the removal of the Z-lines led to a rapid disappearance of the ghosts. Similarly, pre-incubation of the ghosts with the same ratio of HMM S-1 to myosin in a high ionic strength solution (to ensure full penetration of myosin into the thin filament network), followed by washing with rigor solution and digestion of the Z-lines, results

in a rapid (within a few seconds) disappearance of the ghosts. It is also conceivable that the digestion of the Z-lines by trypsin in the presence of HMM is much slower than that in the presence of HMM S-1 (the larger HMM could, for instance, sterically block access of trypsin to the Z-lines). That this could not be the reason for the stability of ghosts pre-incubated with HMM was demonstrated by the fact that the addition of as little as $1 \mu\text{M}$ MgATP (in the presence of 4 mM phosphocreatine and 0.4 mg ml^{-1} creatine kinase) to such ghosts after treatment with trypsin led to their instantaneous disappearance. These control experiments clearly show that it is not the contaminating myosin in the HMM preparation or the incomplete removal of the Z-lines that is responsible for the maintenance of the structural integrity of the ghosts.

It is similarly possible to demonstrate the cross-linking ability of the indigenous myofibrillar myosin. Perfusion of the myofibril with a solution containing 0.6 M KCl, 5 mM Na-phosphate buffer, pH 7.0, causes the thick filaments to dissociate into presumably individual myosin molecules. Z-lines are then removed by the above solution containing, in addition, 0.5 mg ml^{-1} trypsin. Myofibrils thus treated still maintain structural integrity.

Although these experiments do not elucidate which fraction of HMM molecules is involved in the inter-filament cross-linking or whether this type of binding is responsible for the inability of HMM fully to saturate actin binding sites in myofibrillar ghosts¹⁰, they clearly demonstrate the ability of the double-headed myosin moiety to bind different actin filaments *in vivo*. Future research should examine the possibility, which is now more likely, that a similar two-filament interaction occurs during muscle contraction. Whether or not the two heads are

identical, any discussion of the mechanism of contraction will have to take into consideration the capability of a myosin molecule to interact with two different actin filaments.

We thank Rina Levy for technical assistance and the Muscular Dystrophy Association and the US-Israel Binational Foundation for financial support. J. B. is an Established Investigator of the American Heart Association.

Received 4 March; accepted 23 March 1981.

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Thromboxane and prostacyclin release from ischaemic myocardium in relation to arrhythmias

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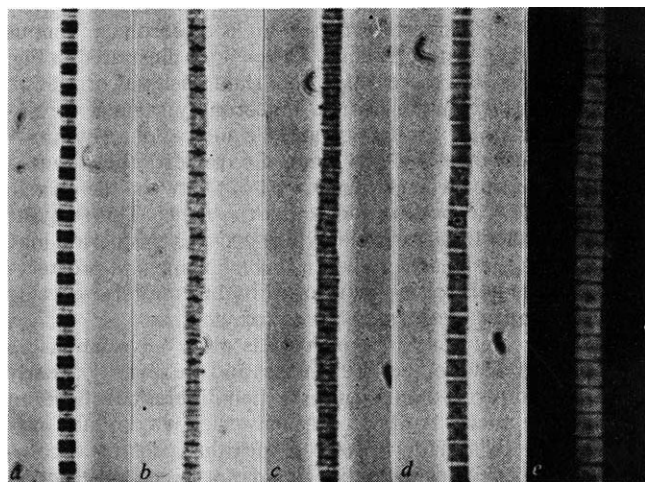


Fig. 1 Myofibrils were obtained by homogenizing glycerinated fibres in rigor solution (in a Sorvall Omni-Mixer for 6 s at speed setting 7, in ice). The myofibrils were filtered through gauze and suspended in a rigor solution containing 0.1 mM sodium azide and 1 mg ml^{-1} phenylmethylsulphonylfluoride. The myofibril bathing solutions were changed by applying a drop of the appropriate solution at one end of the coverslip and soaking it up with blotting paper at the opposite end. All experiments were done at room temperature using the Zeiss Axiomat microscope, $\times 100$ Planapochromat objective, N.A. = 1.3. The phase retardation of the diffraction ring was $-\pi/2$ and its amplitude transmission 0.1. *a*, Intact myofibril in rigor solution; *b*, the same myofibril after myosin extraction with the modified Hasselbach-Schneider solution containing 0.5 M KCl, 0.1 M Na-phosphate buffer, pH 7.0, 5 mM MgCl_2 and 10 mM Na-pyrophosphate. This solution was freshly prepared before each experiment. *c*, The same myofibril after perfusion with a rigor solution. *d*, The same myofibril after removal of the Z-line using rigor solution containing 0.5 mg ml^{-1} trypsin, and a thorough wash with the rigor solution. *e*, Fluorescence image of *d*. The HMM in this experiment was labelled with 2 mol iodoacetamido-fluorescein per mol HMM by the method described elsewhere¹⁰.

There has been much interest in the possible physiological and pathophysiological roles of myocardial prostaglandins¹. One particularly intriguing suggestion² is that they may be capable of protecting the heart against arrhythmogenic stimuli. There is certainly no doubt that some prostaglandins can protect against a variety of arrhythmias induced either chemically or by coronary artery ligation^{2–4}. For example, PGE_2 and prostacyclin are effective against the early post-infarction arrhythmias that result from acute coronary artery ligation in anaesthetized dogs³ and rats⁴. In a previous study^{5,6} we could find no evidence that PGE_2 (or $\text{PGF}_{2\alpha}$) was released from the canine myocardium at a time when ventricular ectopic activity was pronounced. We now demonstrate that, in contrast, both thromboxane and prostacyclin are released from the acutely ischaemic myocardium and that the balance between thromboxane and prostacyclin release in the period immediately following coronary artery ligation is related to the occurrence of arrhythmias.

The studies were performed in greyhounds (21–34 kg) anaesthetized with chloralose (80 mg per kg, intravenously) following induction with sodium thiopentone (25 mg per kg, intravenously). The animals were respired with oxygen using a positive pressure ventilation pump and catheters were placed in the aorta, coronary sinus, left ventricle and pulmonary artery under fluoroscopic control. They were used for blood sampling, pressure measurement and for the determination of cardiac output by thermodilution. Following a left thoracotomy the heart was suspended in a pericardial cradle and a ligature placed around the left anterior descending coronary artery. The vein adjacent to this artery was catheterized using the Seldinger technique such that the tip lay (after coronary artery ligation) within the ischaemic region. Blood samples were taken from the various sites 5 min before acute coronary artery ligation and at 2, 10 and 30 min afterwards. They were analysed for blood gases and pH⁷ and also for thromboxane B_2 and 6-keto $\text{PGF}_{1\alpha}$ (the major breakdown products of thromboxane A_2 and prostacyclin, respectively), using radioimmunoassay techniques developed by us^{6,8}, based on the methods of Dray *et al.*⁹.

Blood samples for thromboxane and prostacyclin assays were immediately placed in plastic tubes containing indomethacin and EDTA and kept on ice until centrifugation (not more than

40 min later) at 2,000g for 10 min. The plasma was removed and stored at -20°C until assay. After acidification, aliquots of plasma were extracted with redistilled ethyl acetate and these extracts were then evaporated to dryness under reduced pressure at 37°C . The recovery of thromboxane B_2 or 6-keto $\text{PGF}_{1\alpha}$ was monitored using tritiated internal standards. Sample extracts and standards were then redissolved in phosphate-buffered saline and tritiated thromboxane B_2 or 6-keto $\text{PGF}_{1\alpha}$ (NEN) was added followed by the appropriate specific antibody (Pasteur Institute). After overnight incubation at 4°C , the antibody-bound and free prostaglandins were separated using dextran-coated charcoal. An aliquot of the antibody-bound fraction was placed in Biofluor scintillant and counted in a Packard Tri-Carb 460 liquid scintillation counter. With the above procedures the detection limits were 15 and 40 pg for thromboxane B_2 and 6-keto $\text{PGF}_{1\alpha}$, respectively.

Acute coronary artery ligation resulted in electrocardiographic and local metabolic changes⁷. These animals exhibited varying numbers of cardiac arrhythmias ranging from 5 to 1,085 ventricular ectopic beats in the first 30 min after ligation. Three animals out of 12 died in ventricular fibrillation during this time.

There was no evidence of myocardial thromboxane release in the control state (arterial, coronary sinus and local coronary venous concentrations of 181 ± 43 , 188 ± 50 and 202 ± 38 pg ml^{-1} , respectively). After coronary artery ligation, however, there was an immediate and marked increase in the thromboxane B_2 concentration in local coronary venous blood (Fig. 1a), evident 2 min after ligation and peaking at 10 min; by 30 min thromboxane values were returning towards control. After ligation there were no significant changes in the concentration of thromboxane B_2 in arterial or coronary sinus blood, which drains the essentially normal myocardium (Fig. 1a). These results indicate, therefore, that coronary artery ligation results in the early release of thromboxane only from the ischaemic region.

Despite marked variations in 6-keto $\text{PGF}_{1\alpha}$ concentrations between individual animals (mean arterial concentration before ligation, 462 ± 166 pg ml^{-1}), there was evidence that 6-keto $\text{PGF}_{1\alpha}$ was released from the myocardium in the control state, as the coronary sinus concentrations were higher than the arterial values in each animal, the difference ranging from 133 to 364 pg ml^{-1} , mean $\pm$ s.e.m. 242 ± 28 , $n = 9$. After coronary artery ligation there was a further myocardial release of 6-keto $\text{PGF}_{1\alpha}$ but, in contrast to thromboxane release, this occurred rather more gradually and from both the ischaemic and normal regions of the left ventricular wall (Fig. 1b).

Close examination of the results in each individual animal revealed an interesting pattern. At 2 min post-ligation, the

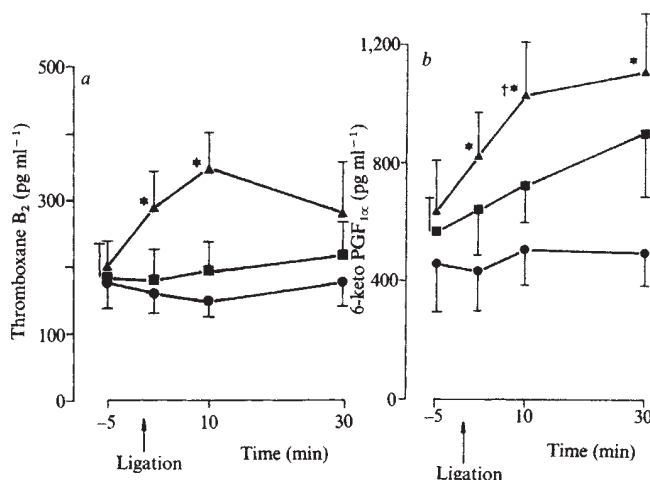


Fig. 1 Thromboxane B_2 (a) and 6-keto $\text{PGF}_{1\alpha}$ (b) values in the aorta (●), coronary sinus (■) and local coronary vein (▲) before and 2, 10 and 30 min after coronary artery ligation. Each value represents the mean $\pm$ s.e.m., $n = 9$. * $P < 0.05$ (venous vs arterial), † $P < 0.05$ (venous vs sinus), dependent t -test.

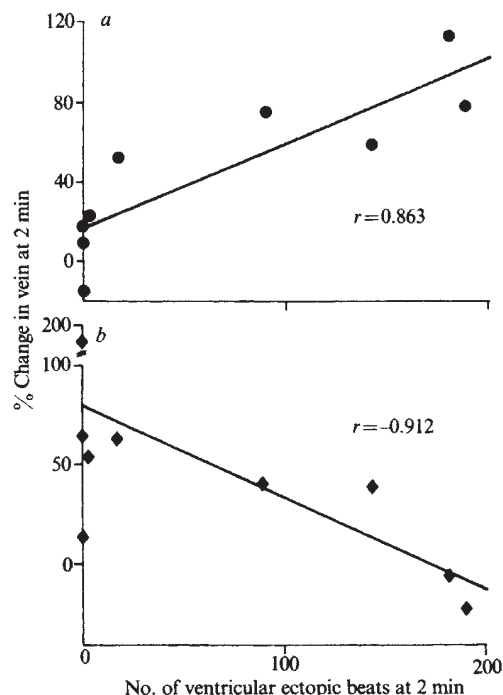


Fig. 2 Correlation between the % change in thromboxane B_2 in the local coronary vein 2 min post-ligation and the number of arrhythmias which had occurred by that time (a) and similarly between the % change in 6-keto $\text{PGF}_{1\alpha}$ and arrhythmias (b).

animals which exhibited the most ventricular ectopic activity also showed the largest increases in local coronary venous thromboxane B_2 concentrations. This point is illustrated in Fig. 2, which shows that there was a significant positive correlation ($r = 0.863$) between the change in coronary venous thromboxane B_2 at 2 min post-ligation and the number of arrhythmias which had occurred by this time. Conversely, there was a significant negative correlation ($r = 0.912$) between the change in 6-keto $\text{PGF}_{1\alpha}$ concentrations and arrhythmias. This suggests that the number of arrhythmias which occur in this period may be related to the balance between thromboxane and prostacyclin release, a balance in favour of thromboxane release being associated with a greater number of arrhythmias.

The most likely explanation for this observed relationship between the release of these substances and the severity of early post-infarction arrhythmias concerns their differential effects on the coronary microcirculation. Thromboxane may reduce oxygen delivery to already hypoxic myocardial cells by a combination of local vasoconstriction¹⁰ and mechanical obstruction resulting from platelet aggregation. Prostacyclin, on the other hand, would both inhibit platelet aggregation and dilate small coronary vessels. It is not known whether thromboxane A_2 has direct arrhythmogenic properties but prostacyclin has been shown to have anti-arrhythmic activity in the dog⁴. These results suggest that a stable prostacyclin analogue and/or a specific thromboxane synthesis inhibitor might prove beneficial in the early stages of acute myocardial infarction.

Supported by the Scottish Hospitals Research Trust.

Received 8 January; accepted 19 March 1981.

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Sodium current depression by lidocaine and quinidine in isolated ventricular cells

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A number of local anaesthetics are now widely used as cardiac antiarrhythmic agents, but the cellular basis for these actions in the heart remains controversial¹. One hypothesis is that agents such as lidocaine and quinidine selectively depress the fast inward current, I_{Na} . We have now tested this hypothesis using recently developed techniques for isolating and voltage clamping rat ventricular cells. Our results provide direct evidence that therapeutic doses (10–20 μ M) of lidocaine or quinidine strongly inhibit I_{Na} , and give some clues about the mechanism of this inhibition.

Weidmann² first reported that the local anaesthetic cocaine depresses the maximum upstroke velocity (V_{max}) of the action potential in sheep Purkinje fibres; his results also suggested that this effect was produced by a voltage-dependent shift in the inactivation mechanism governing I_{Na} . Johnson and MacKinnon³ and Heistracher⁴ later showed that quinidine sulphate depressed V_{max} in atrial tissue and papillary muscle. Their results emphasized that the amount of depression of V_{max} was strongly dependant on stimulus frequency. A similar frequency-dependent, or use-dependent, depression of V_{max} by lidocaine, quinidine and various other local anaesthetic agents in cardiac tissue has now been reported^{5,6}. However, V_{max} in most experimental tests provides only an indirect and nonlinear measure of the maximum available sodium conductance, $\bar{g}_{Na}$ (refs 7–11).

A reduction in I_{Na} by local anaesthetics has been previously observed in voltage-clamp experiments done in squid axon, frog node of Ranvier, and frog skeletal muscle (see ref. 12). These data show that local anaesthetics (0.1–5 mM at pH 7.2–7.4) produce a large tonic block as well as a use-dependent inhibition of I_{Na} . Most of the data from these tissues are consistent with a model (see ref. 12) of use-dependence in which the cationic drug molecules bind to a site within the sodium channel, and this drug binding is enhanced when the I_{Na} channels are in the inactivated state. There is no direct evidence that this mechanism is applicable to cardiac muscle, therefore we have attempted to study the actions of lidocaine and quinidine on I_{Na} in single cells derived from rat ventricular tissue. These two drugs were chosen because the kinetics of their use-dependent inhibition of I_{Na} has been postulated to be very different⁵. Our initial experiments were restricted to semiquantitative measurements of I_{Na} at normal pH in the presence of therapeutic doses ($\sim 10^{-5}$ M) of these two drugs. Emphasis was placed on possible drug-induced changes in the inactivation of I_{Na} .

Isolated cells from the ventricle of adult rats were prepared for internal perfusion and voltage clamp of these cells, using the procedure of Powell and Twist¹³. A suction pipette technique^{14,15} which was developed in this laboratory was utilized. In some experiments, two suction pipettes were used to achieve more effective series resistance compensation. As the resulting clamp data were qualitatively similar to those obtained using a single suction pipette with series resistance compensation, all results were combined. The superfusion solution (pH 7.4) contained 44 mM Na-isethionate, 52.4 mM Cs aspartate, 2.8 mM NgSO_4 , 1.1 mM MgCl_2 , 1.1 mM CaCl_2 , 5.5 mM glucose, 80 mM sucrose and 8 mM Tris base. Thus, $[\text{Na}]_0$ was

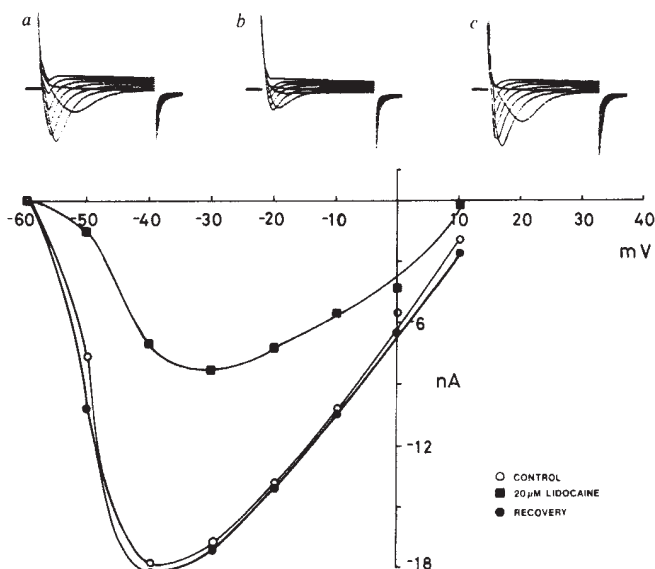


Fig. 1 Effects of lidocaine-HCl (20 μ M) on I_{Na} in isolated cells from the rat ventricle. *a–c*, Are superimposed current records resulting from 10-ms depolarizations (+20–+90 mV) applied at 0.1 Hz from a holding potential of –80 mV. *b*, Shows the reduction in I_{Na} after exposure to the drug for 10 min. Note the marked reduction in I_{Na} with relatively little change in steady background current. The bottom part of the figure shows corresponding I – V relationships for I_{Na} . Each data point was obtained by subtracting the maximum inward current from the steady background current recorded at the end of the depolarizing pulse. ■, The I – V relationship obtained for lidocaine (20 μ M); ○ and ● represent control and recovery tests, respectively.

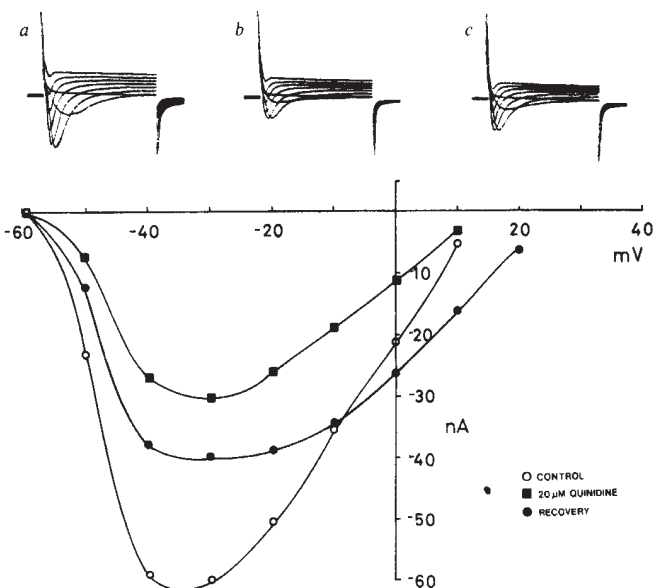


Fig. 2 Effects of quinidine-HCl (20 μ M) on I_{Na} in isolated cells from the rat ventricle. For details of experimental protocol, see Fig. 1 legend. *b*, And the I – V relationship (■) indicate the voltage-clamp data obtained in the presence of the drug.

reduced to about one-third normal, and sodium was the only permeant monovalent cation in the external solution. The solution which perfused the fibre internally (pH 7.2) consisted of 135 mM Cs aspartate, 17 mM NaHCO_3 , 5 mM glucose and 2 mM Tris base. Transmembrane voltage and current waveforms were displayed on a storage oscilloscope and digitized every 2 μ s using a Nicolet 1170 digital oscilloscope. Digitized records were stored on tape (Kennedy 9700 digital tape recorder) for off-line analysis. All experiments were done at room temperature.

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Figure 1 shows records of I_{Na} obtained in the presence (*b*) and absence (*a* and *c*) of 20 μ M lidocaine-HCl. A plot of the I - V relation for I_{Na} is shown in the bottom part of Fig. 1. In this and all subsequent analyses, I_{Na} was measured as the ΔI between the peak of I_{Na} and the steady level of current at the end of a 10-ms

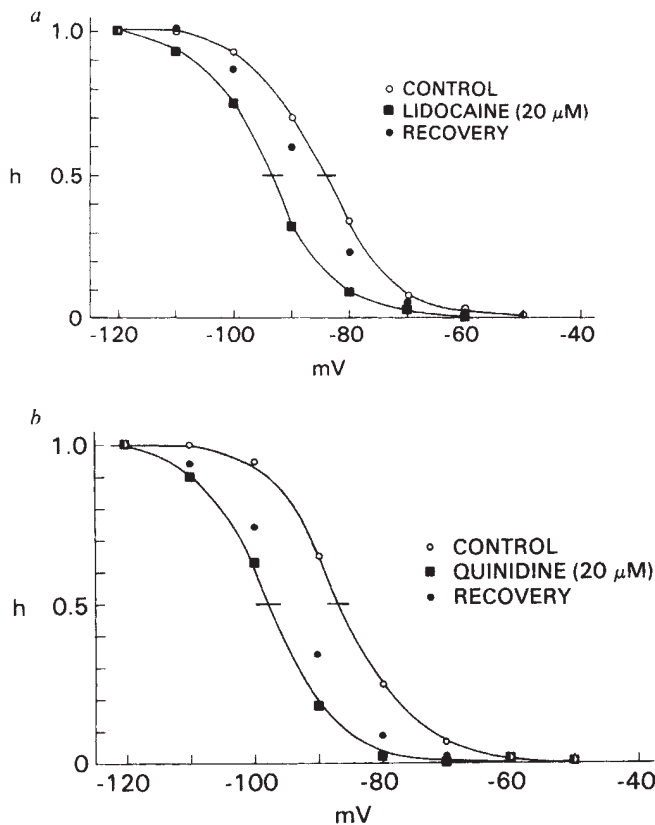


Fig. 3 *a*, Effect of lidocaine-HCl (20 μ M) on steady-state inactivation of I_{Na} . Voltage dependence of the inactivation process was measured by applying a series of paired pulses at 0.1 Hz. A 500-ms prepulse of variable magnitude (-140 to -50 mV) was followed immediately by a standard test pulse of +40 mV for 10 ms. Points on the curves ($\circ$, control; $\blacksquare$, lidocaine-HCl and $\bullet$, recovery) represent the normalized peak inward currents corresponding to each prepulse voltage level. The horizontal lines represent 50% inactivation for the control and drug curves. *b*, Effect of quinidine-HCl (20 μ M) on steady-state inactivation of I_{Na} . Same protocol as for *a*. Note that quinidine also produces a voltage-dependent shift in the h curve. Schwarz *et al.*²¹ have drawn attention to the complexities of this measurement when testing agents which have a significant use-dependent effect. In their view, the protocols used in these experiments measure the h curve of those Na channels which are not blocked at rest.

depolarizing voltage clamp pulse. Possible use-dependent depression of I_{Na} was minimized by applying clamp pulses at 0.1 Hz, after the quiescent preparation had been exposed to the drug for 3–5 min. It is apparent from Fig. 1 that lidocaine-HCl strongly reduces I_{Na} , and that this effect is selective and fully reversible after a 10–20-min wash period. Similar results (50–80% tonic or resting reduction in I_{Na}) were obtained in six additional experiments.

Figure 2 shows data from an identical study of the effects of 20 μ M quinidine-HCl on I_{Na} . A selective ($\sim 70\%$) tonic reduction in the peak I_{Na} was observed in each of seven experiments. However, we did not obtain full reversibility before the preparation began to deteriorate (~ 20 min).

We next tested whether this decrease in I_{Na} was produced by drug-induced changes in the inactivation variable which controls I_{Na} . Figure 3 shows that both lidocaine-HCl (*a*) and quinidine-HCl (*b*) shifted the steady-state inactivation curve (produced by 500-ms prepulses) to more negative or hyperpolarized potentials. When such inactivation curves are normalized, the mean voltage shift ($\pm$ s.e.m.) measured at the midpoint of the curve is: lidocaine-HCl (10.4 ± 1 mV, $n = 5$); quinidine-HCl (13.5 ± 1.9 mV, $n = 5$). These results are similar to the data obtained by Weidmann². Chen *et al.*¹⁶, however, found no quinidine-induced voltage dependent shift in I_{Na} inactivation as measured by $\dot{V}_{max}$.

The finding that most antiarrhythmic agents prolong the refractory period in ventricular tissue has been attributed to their ability to prolong the recovery of I_{Na} from inactivation¹⁷. We therefore studied the recovery of I_{Na} in the presence and absence of lidocaine-HCl and quinidine-HCl. Recovery at -80 mV was assessed by measuring I_{Na} in response to paired 40-mV, 40-ms depolarizing pulses separated by intervals varying from 20 to 500 ms. Each two-pulse sequence was followed by a 10–15-s rest period. For lidocaine-HCl (20 μ M) the half time of recovery was prolonged from 87 ± 14 to 125 ± 14 ms ($n = 6$); for quinidine-HCl the half time of recovery was slowed from 42 ± 5 to 70 ± 9 ms ($n = 7$). The half time of the recovery process was chosen as a measure because the I_{Na} reactivation does not follow a simple exponential time course in these rat ventricular cells¹⁸.

The final series of experiments (see Fig. 4) was done to assess the extent to which frequency-dependent or use-dependent inhibition of I_{Na} contributes to the observed reduction in I_{Na} . Trains of 20 depolarizing voltage clamp pulses (-80 to -40 mV for 40 ms) were applied at frequencies ranging from 0.1 to 5 Hz. No measurable use-dependent effects were observed in either drug when the pulsing frequency was less than 0.3 Hz. At 5 Hz a small use-dependent inhibition was observed. Note that in Fig. 4*a* the resting or tonic blockade by lidocaine-HCl is 40% of the control I_{Na} whereas frequency-dependent blockade produced an additional 10% inhibition. Similarly, Fig. 4*b* shows that quinidine produces 60% tonic block and that the 5-Hz train produces only an additional 8% inhibition. These results contrast with the previously reported marked modulation of

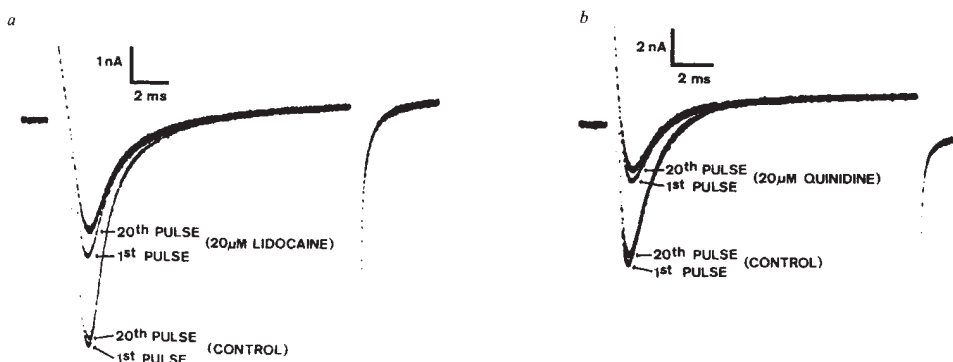


Fig. 4 *a*, Frequency- or use-dependent effects of lidocaine-HCl (20 μ M) and *b*, quinidine-HCl (20 μ M). Trains of 20 depolarizing voltage clamp pulses (-80 mV to -30 mV for 10 ms) were applied at 5 Hz. The current records in response to pulse no. 1 and pulse no. 20 were superimposed using a storage oscilloscope. When control records has been obtained, the drug was applied to the quiescent preparations, and after 5–10 min the 20 pulses were again applied. Both drugs produced small use-dependent suppression of I_{Na} . Note however, that even in the control test, the I_{Na} in response to pulse 20 was smaller than that elicited by pulse 1.

$V_{\max}$ by lidocaine and quinidine^{5,6}, which implied much larger use-dependent effects on I_{Na} in cardiac muscle. Moreover, they suggest that relatively low doses of two antiarrhythmic agents inhibit I_{Na} in a similar fashion: both produce a large 'resting' or tonic inhibition. The inactivation curve is shifted to the left and an additional use-dependent inhibition is observed although the use-dependent effect is quite small in these experimental conditions.

Before the relative importance of use-dependent compared with tonic blockade of I_{Na} can be assessed conclusively, additional data must be collected in conditions in which the pH, the holding potential and the potential to which the cells are

depolarized are widely varied. Analogous experiments in squid axon¹⁹ have led to the suggestion that there may be two separate receptors for the resting or tonic block and the frequency-dependent blocking. In addition, the anaesthetic-induced changes in outward current(s) in the heart^{20,22} must be studied quantitatively before the basis of these antiarrhythmic actions can be understood fully.

We thank Dr Trevor Powell for demonstrating his technique of rat cell preparation and Ester Lee for technical assistance. This research was supported by a NSF fellowship to J.R.H. and grants from the American Heart Association (77-691 to W.G.) and USPHS (HL-25409 to W.G.; HL-25145 to A.M.B.).

Received 2 October 1980; accepted 12 March 1981.

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Calcitonin selectively stimulates 25-hydroxyvitamin D₃-1 α -hydroxylase in proximal straight tubule of rat kidney

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We have reported previously that the 25-hydroxyvitamin D₃ (25(OH)D₃)-1 α -hydroxylase (1 α (OH)ase) activity was localized exclusively in the proximal convoluted tubules (PCT) in mature vitamin D-deficient rats and that the enzyme activity was largely abolished by parathyroidectomy in the vitamin D-deficient rats with presumed secondary hyperparathyroidism¹. However Akiba *et al.*² found enzyme activity in both PCT and the proximal straight tubules (PST) of the fetal rabbit kidney. Parathyroid hormone and calcitonin, when given *in vivo*, can stimulate the production of 1 α , 25(OH)₂D₃ from 25(OH)D₃ in vitamin D-deficient rats^{3–6}, and, moreover, their *in vivo* effects on the 1 α (OH)ase in thyroparathyroidectomized vitamin D-deficient rats are additive⁶, a finding consistent with different sites of action of these two hormones. In addition, plasma calcitonin levels are elevated in the fetus of several mammalian species^{7–10}, while they are likely to be low in hypocalcaemic vitamin D-deficient rats. From these observations, we hypothesized that calcitonin could be responsible for stimulating enzyme activity in the PST while parathyroid hormone primarily activates 1 α (OH)ase in the PCT. To test this hypothesis, we examined the effect of calcitonin on the 1 α (OH)ase activity in defined nephron segments of vitamin D-deficient rats. We report here that the hormone selectively stimulates 1 α (OH)ase activity in the PST, whereas this enzyme activity is undetectable in control vitamin D-deficient animals.

Male weanling Holtzman rats were fed a vitamin D-deficient diet containing 0.45% calcium and 0.3% phosphorous for 3–7 weeks¹. Vitamin D-deficient rats were given graded doses of synthetic salmon calcitonin (Armour) and calcium gluconate, 200 mg per kg subcutaneously every 2 h; the latter was given to

prevent a further fall in plasma calcium concentration. The rats were killed 2 hours after the last injection of calcitonin. Control vitamin D-deficient animals received the vehicle for calcitonin (0.9% NaCl) either with or without calcium gluconate. Plasma concentrations of calcium and inorganic phosphate in vitamin D-deficient rats, measured at the time of death, were 6.1 ± 0.4 and 5.8 ± 0.3 mg dl⁻¹, respectively, with no differences between those receiving and not receiving calcium gluconate; calcium and inorganic phosphate values in calcitonin-treated rats were 5.8 ± 0.5 and 5.4 ± 0.7 mg dl⁻¹, respectively. The conversion of ³H-25(OH)D₃ to ³H-1 α , 25(OH)₂D₃ was determined in each group of defined tubular segments, microdissected from collagenase-treated kidney slices and incubated in 20 μ l Hanks' solution containing 5 mM pyruvate, 8.3 mM glucose, 10 mM HEPES, pH 7.4, and 0.5 μ M ³H-25(OH)D₃ (see Fig. 1). Each incubation contained the specific segments from one animal, and only one incubation was prepared for any given segments from each animal. The incubations, carried out at 37 °C for 60 min, were terminated by the addition of 50 μ l chloroform/methanol (1:2, v/v) followed by lipid extraction¹¹.

Lipid extracts were subjected to the silica gel 60 TLC; the radioactive peaks were re-extracted and underwent HPLC to identify the metabolites of ³H-25(OH)D₃ (ref. 1). The PCT of control rats and both the PCT and PST of calcitonin-treated rats produced a single radioactive peak from ³H-25(OH)D₃; this peak co-migrated with authentic 1 α , 25(OH)₂D₃ in both TLC and HPLC. The amounts of 1 α , 25(OH)₂D₃ produced, expressed in fmol ³H-1 α , 25(OH)₂D₃ produced per h per mm

Table 1 1 α (OH)ase activity along the nephron of vitamin D-deficient rats given calcitonin

Nephron segment	n	Tubular length per incubation (mm)	1 α (OH)ase activity (fmol mm ⁻¹ h ⁻¹)
Glomerulus	4	50–100*	ND
Proximal convoluted tubules	4	21–38	0.65 $\pm$ 0.08
Proximal straight tubules	4	23–26	0.69 $\pm$ 0.09
Medullary thick ascending limb	4	39–85	ND
Cortical thick ascending limb	4	23–78	ND
Distal convoluted tubules	4	19–35	ND
Collecting tubules	4	32–62	ND

Defined nephron segments were dissected from kidney slices of vitamin D-deficient rats 8 h after four injections of calcitonin, 20 MRCU per kg every 2 h and assayed for 1 α (OH)ase activities as described in Fig. 1 legend. Enzyme activities in kidneys of vitamin D-deficient rats without calcitonin treatment were 0.70 ± 0.05 fmol mm⁻¹ h⁻¹ in the PCT and undetectable in all other nephron segments including the PST¹. Results are the mean $\pm$ s.e.m. ND, not detectable or < 1 fmol per incubation.

* The number of glomeruli per incubation.

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Table 2 Effect of calcitonin on $1\alpha(\text{OH})\text{ase}$ in PCT and PST of thyroparathyroidectomized vitamin D-deficient rats

	$1\alpha(\text{OH})\text{ase}$ activity (fmol $\text{mm}^{-1} \text{h}^{-1}$)	
	PCT	PST
Control	0.17 ± 0.10	ND
With calcitonin treatment	0.16 ± 0.06	0.73 ± 0.11

Rats were fed a vitamin D-deficient diet containing 0.45% calcium and 0.3% phosphorous for 4–6 weeks from weanling, then supplemented with additional 0.75% calcium (total 1.2% calcium) for 3–5 days before thyroparathyroidectomy done under light anaesthesia. Starting 16 h after surgery, the rats received four successive subcutaneous injections of either calcitonin, 50 MRC U per kg, and calcium gluconate, 200 mg per kg, or the vehicle (control) every 2 h; they were killed 24 h after the surgery. Results are the mean $\pm$ s.e.m., $n = 4$ –6. ND, not detectable or $< 0.05 \text{ fmol mm}^{-1} \text{h}^{-1}$.

tubular length or per glomerulus, were calculated from data obtained by TLC and the specific activity of ^3H - $25(\text{OH})\text{D}_3$ corrected for recovery (82–92%) and counting efficiency (43–52%)¹. The results were expressed as the mean $\pm$ s.e.m. and analysed by the t -test.

As shown in Fig. 1a and b, the administration of calcitonin to vitamin D-deficient rats resulted in a marked stimulation of $1\alpha(\text{OH})\text{ase}$ activity in PST which was undetected in control rats. This stimulatory effect of calcitonin appeared at 4–6 h and reached a plateau at 8 h (Fig. 1a); maximal stimulation was obtained with 20 MRC units per kg every 2 h (Fig. 1b). Table 1 shows the distribution of the enzyme activity along the nephron segments from hormone-treated rats. $1\alpha(\text{OH})\text{ase}$ activities in the PCT in rats with and without calcitonin treatment were 0.65 ± 0.08 and $0.70 \pm 0.05 \text{ fmol mm}^{-1} \text{h}^{-1}$, respectively. There was no stimulation of the enzyme by calcitonin in other nephron segments tested, including glomeruli, medullary and cortical thick ascending limbs of Henle's loop, distal convoluted tubules and collecting tubules.

These results clearly demonstrate that calcitonin selectively stimulated $1\alpha(\text{OH})\text{ase}$ activity in the PST, and that the enzyme activity was undetectable in control vitamin D-deficient rats.

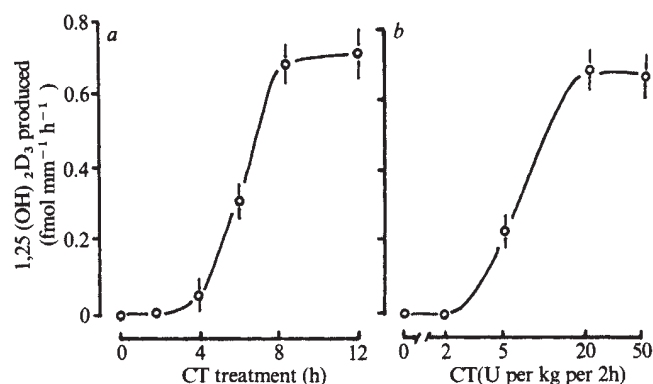


Fig. 1 Time course (a) and dose response (b) of the effects of calcitonin (CT) on the $1\alpha(\text{OH})\text{ase}$ activity in the PST of vitamin D-deficient rats. Under pentobarbital anaesthesia, 40 mg per kg, the left kidney was perfused with chilled Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 8.3 mM glucose, 0.1% bovine serum albumin (fraction V, Sigma) and 0.1% collagenase (type I, Sigma). Kidney slices were then incubated for 30 min at 30°C in the same buffer with a constant bubbling with a gas mixture of 95:5% = $\text{O}_2:\text{CO}_2$. After rinsing the slices three times with 30–50 ml of ice-cold modified Hanks' solution (in mM: NaCl, 137; KCl, 5.2; Na_2HPO_4 , 0.34; KH_2PO_4 , 0.44; CaCl_2 , 1.0; MgCl_2 , 0.49; MgSO_4 , 0.42; and NaHCO_3 , 4.2; pH 7.4), the PST were dissected in the Hanks' solution at 0°C under a stereomicroscope. Tubular segments of known length were incubated for 60 min at 37°C in 20 μl of modified Hanks' solution containing 5 mM pyruvate, 8.3 mM glucose and 10 mM HEPES, pH 7.4, with $0.5 \mu\text{M}$ [26, 27- ^3H]- $25(\text{OH})\text{D}_3$ (specific activity 22.3 Ci mmol^{-1}) or [23, 24(n)- ^3H]- $25(\text{OH})\text{D}_3$ (specific activity 102 Ci mmol^{-1}), both isotopes from Amersham) with room air as a gas phase as reported previously¹. Lipid extracts¹¹ were applied to silica gel 60 TLC and ^3H - $1,25(\text{OH})_2\text{D}_3$ produced, verified by HPLC¹, were calculated in fmol per mm tubular length per h based on the specific activity of ^3H - $25(\text{OH})\text{D}_3$, corrected for recovery (82–92%) and counting efficiency (43–52%). a, Calcitonin, 20 MRC U per kg, was given every 2 h. b, Calcitonin was given at doses indicated on the abscissa every 2 h and rats were killed at 8 h.

However, these results do not exclude a possible stimulatory effect of calcitonin on the enzyme activity in the PCT, as it may have failed to stimulate $1\alpha(\text{OH})\text{ase}$ activity in the PCT because the activity was maximally activated by the hyperparathyroidism of vitamin D deficiency. To test this possibility, we examined the effect of calcitonin on the enzyme activity in both the PCT and PST of thyroparathyroidectomized vitamin D-deficient rats. As shown in Table 2, calcitonin stimulated the $1\alpha(\text{OH})\text{ase}$ in the PST but not in the PCT, indicating the specificity of the stimulation of $1\alpha(\text{OH})\text{ase}$ in the PST. Our results are in accord with *in vivo* observations by Horiuchi *et al.* that calcitonin stimulated the $1\alpha(\text{OH})\text{ase}$ independently of parathyroid hormone and that the effects of the two hormones are additive⁴. Furthermore, it is evident from our data that calcitonin and parathyroid hormone can act on anatomically and functionally distinct segments of the nephron.

Parathyroid hormone is believed to stimulate $1\alpha(\text{OH})\text{ase}$ by stimulating adenylate cyclase^{5,6}. As calcitonin also stimulates adenylate cyclase in the kidney, we examined the distribution of calcitonin-sensitive adenylate cyclase along the nephron both from vitamin D-deficient and normal, vitamin D-replete rats. As shown in Table 3, the hormone did not stimulate adenylate

Table 3 Calcitonin-sensitive adenylate cyclase activity along the rat nephron

Nephron Segments	Adenylate cyclase activity, cyclic AMP produced (fmol per mm per 30 min)			
	Normal		Vitamin D-deficient	
	-CT	+CT	-CT	+CT
Proximal convoluted tubules	4.3 ± 1.5	8.4 ± 2.1	NT	NT
Proximal straight tubules	12.2 ± 2.0	16.7 ± 3.1	5.6 ± 3.0	6.0 ± 2.6
Medullary thick ascending limb	46.9 ± 1.5	$220.1 \pm 19.4^*$	NT	NT
Cortical thick ascending limb	40.9 ± 19.3	$433.6 \pm 54.6^*$	37.1 ± 10.4	$402.0 \pm 53.6^*$
Distal convoluted tubules	9.5 ± 2.3	$627.9 \pm 104.2^*$	NT	NT
Collecting tubules	11.5 ± 0.6	$75.0 \pm 4.7^*$	NT	NT

Adenylate cyclase activities in defined nephron segments were assayed with (+CT) or without (-CT) the addition of calcitonin in the incubation medium, by the method of Imbert *et al.*¹⁴ with modifications¹⁵. Results are the mean $\pm$ s.e.m. of three to six incubations, and concentration of calcitonin used was 0.1 U ml^{-1} in all incubations except for PCT and PST of normal rats where it was 1.0 U ml^{-1} . NT, not tested.

*Compared with -CT, $P < 0.01$.

cyclase in the PST of either normal or vitamin D-deficient rats, but did stimulate the adenylate cyclase activity in the cortical thick ascending limb of Henle's loop, as has been reported previously¹². These data strongly suggest that calcitonin may stimulate $1\alpha(\text{OH})\text{ase}$ in the PST of vitamin D-deficient rats by a mechanism independent of adenylate cyclase activation. The additive effects of CT and cAMP on $1\alpha, 25(\text{OH})_2\text{D}_3$ production in thyroparathyroidectomized vitamin D-deficient rats⁶ are consistent with such a postulate. Also, Larkins *et al.*¹³ found that CT can stimulate $1\alpha(\text{OH})\text{ase}$ in chick kidney tubules *in vitro*.

Our results provide a plausible explanation for the presence of $1\alpha(\text{OH})\text{ase}$ in the PST of the kidney of the fetus² whose circulating calcitonin levels are probably elevated^{7–10}, but not in the kidney of mature, vitamin D-deficient rats¹, which are likely to have low plasma calcitonin levels. The data also suggest a possible physiological role, hitherto unrecognized, of calcitonin in vitamin D metabolism. We propose that calcitonin may be able to recruit additional tubular cells, that is those in the PST, to produce $1\alpha, 25(\text{OH})_2\text{D}_3$; these cells are potentially capable of producing this sterol but may be latent in vitamin D deficiency and are recruited in special circumstances when the need for calcium is markedly enhanced, as may occur in the fetus.

We thank Dr Jack W. Coburn for valuable discussions. This work was supported by the Veterans Administration and grants from the USPHS (AM-21351 and AM-14750) and American

Diabetes Association. S. T. is an Advanced Research Fellow of American Heart Association, Greater Los Angeles Affiliate.

Received 16 December 1980; accepted 13 March 1981.

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Maintenance of blood pressure by the renin-angiotensin system in normal man

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The view that the renin-angiotensin-aldosterone system is a defence mechanism that maintains blood pressure only in conditions of volume depletion or sodium restriction¹ has been supported by studies with saralasin^{2,3}, a competitive inhibitor of angiotensin II, and teprotide¹, an inhibitor of angiotensin converting enzyme that blocks the formation of angiotensin II. However, both saralasin and teprotide will underestimate the role of the renin-angiotensin system because: (1) they have to be given intravenously, thus inhibiting only the short-term effects of circulating angiotensin II; (2) all subjects in these studies were rested for some time before infusion^{1–3}; and (3) saralasin is an agonist^{4–7} which only inhibits the action of circulating angiotensin II when its level is high. Niarchos *et al.*⁸ demonstrated a drop in blood pressure with teprotide in normal subjects on a sodium intake of 150 mmol per day, but this was partly due to one subject fainting. Therefore, none of these studies clearly shows at what stage of volume depletion the system actively maintains blood pressure. By analogy with other biological systems, a gradual increase in the importance of the renin-angiotensin system as sodium is lost is more likely than an all-or-none phenomenon. Using the recently developed oral inhibitors of angiotensin converting enzyme^{9–11}, it is now possible to inhibit the formation of angiotensin II long-term and study human subjects during normal activity. We have now investigated the effect of one such inhibitor, captopril, in normotensive, healthy, male subjects on a normal sodium intake. Our results demonstrate that the renin-angiotensin system maintains blood pressure in these conditions and that the system is an important controller of aldosterone secretion as well as sodium excretion on a normal sodium intake.

A 3-week study was made of eight normotensive, healthy, male subjects (mean age 22 (21–26) yr), whose diastolic pressure was <80 mm Hg after a 2-month observation period. All subjects were on a fixed sodium intake of 120 mmol sodium per day and 115 mmol potassium per day. During the 3 weeks of

Table 1 Mean values for blood pressure, plasma angiotensin II, plasma renin activity and plasma aldosterone at various times during the study

	Before captopril	Within 2 h of first dose	Fifth day of captopril
Blood pressure (mm Hg)	85.3 ± 0.70	78.6 ± 2.2*	71.6 ± 1.9*
Plasma angiotensin II (pmol l ⁻¹)	25.3 ± 2.3	15.5 ± 1.9*	13.3 ± 1.7*
Plasma renin activity (ng ml ⁻¹ h ⁻¹)	3.3 ± 0.5	5.7 ± 0.7*	41.4 ± 7.1*
Plasma aldosterone (pmol l ⁻¹)	549 ± 64	184 ± 26*	158 ± 14*

Values are mean ± s.e.m.

*P < 0.001 by Student's *t*-test.

this diet all food and drink was supplied by the metabolic ward kitchen. The subjects were not admitted to hospital and were allowed to go about their normal activities although vigorous exercise was discouraged. On the eighth day of the diet, captopril therapy was started, and measurements were made 2 h after the first dose of 25 mg. They were then given 25 mg captopril, three times a day for the first day, increasing on the second day to three 50-mg doses, and on the third and fourth day to 100 mg three times a day. A final dose of 100 mg was given on the morning of the fifth day of captopril therapy. After stopping the captopril, the diet was continued for a further 9 days.

Daily measurements of blood pressure and 24-h urinary sodium and potassium excretion were made. Urinary aldosterone was measured 3 days before, during the 5 days of and for 3 days after captopril therapy. Blood pressure was measured using semi-automatic ultrasound sphygmomanometers with automatic recorders (Arteriosonde, Roche)¹² in the same room, by the same nurse, at the same time of day, except for the 2-h post-captopril measurement. The mean value of five readings at 1–2-min intervals was taken as the blood pressure in that position, except for post-exercise blood pressure, where a single reading was taken 1 min after a standard period of exercise on a treadmill. Pulse rate was measured using a Cambridge 3048 pulse monitor. Measurements of plasma concentrations of angiotensin II, aldosterone, electrolytes and creatinine, plasma renin activity and blood urea were made on the fifth and eighth day before captopril, 2 h after the first dose of captopril and on the fourth and fifth day during captopril treatment. Plasma noradrenaline was measured on the eighth day before, and the fifth day of captopril treatment. All blood was taken without stasis after sitting upright for 5–10 min. Plasma angiotensin II (ref. 13), plasma renin activity¹⁴, plasma¹⁵ and urinary aldosterone were measured by radioimmunoassay, and plasma noradrenaline by radioenzymatic assay¹⁶. All results are reported as mean ± s.e.m. Mean blood pressure was calculated as diastolic pressure + (1/3 × pulse pressure). Statistical analysis was performed using Student's *t*-test.

The results of blood pressure, and plasma angiotensin II, renin activity and aldosterone measurements are shown in

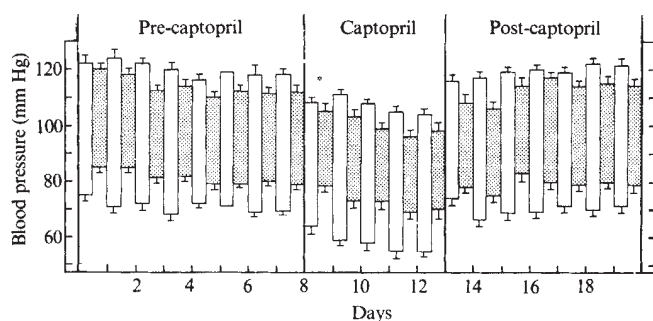


Fig. 1 Average daily supine (open bars) and standing (dotted bars) systolic and diastolic blood pressure (± s.e.m.) in the eight normal subjects on a sodium intake of 120 mmol per day, 7 days before, 5 days of and 7 days after captopril treatment. *2 h after first 25-mg dose of captopril.

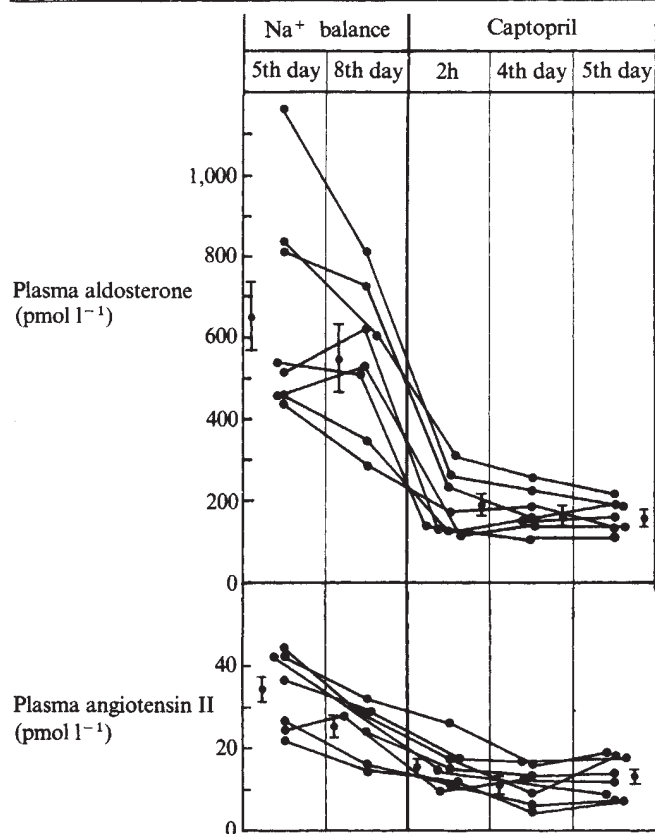


Fig. 2 Mean plasma aldosterone and angiotensin II in the eight normal subjects on a sodium intake of 120 mmol per day on the fifth and eighth day of sodium intake, 2 h after the first 25 mg of captopril and on the fourth and fifth day of captopril.

Table 1. After 5 days of captopril therapy, mean blood pressure had fallen by 16.5% compared with control values before captopril. On stopping captopril, blood pressure returned to the pre-captopril value. Figure 1 shows the average daily systolic and diastolic supine and standing pressure throughout the study. The fall in standing, sitting and post-exercise blood pressure was similar to that found with supine blood pressure. Pulse rate was not altered by captopril. Plasma renin activity rose, whereas plasma angiotensin II and aldosterone levels fell (see also Fig. 2).

Figure 3 shows the urinary aldosterone, potassium, sodium and weight for the 4 days before, the 5 days during and the 4 days after captopril therapy. There was a significant reduction in urinary aldosterone excretion during captopril therapy: mean values are $50.0 (\pm 4.7)$ nmol per 24h 3 days before captopril, and $36.5 (\pm 2.6)$ nmol per 24h ($P < 0.01$) during captopril. All subjects were in a negative sodium balance during captopril treatment, with a mean loss of 142 mmol (± 10.6) of sodium per subject. All subjects lost weight, by the fifth day of captopril, the mean weight loss was 1.15 kg (± 0.31 ; $P < 0.001$).

Plasma noradrenaline, which was $392 (\pm 39)$ pg ml⁻¹ before captopril rose to $712 (\pm 125)$ pg ml⁻¹ ($P < 0.05$) by the fifth day of treatment. Blood urea, plasma electrolytes and plasma creatinine showed no significant change. Plasma potassium rose from $3.9 (\pm 0.1)$ to $4.1 (\pm 0.1)$ mmol l⁻¹ by the fifth day of captopril, but this rise was not significant.

Thus, captopril given for 5 days caused a 16.5% fall in mean blood pressure in these normotensive, healthy subjects on a sodium intake of 120 mmol per day. There is controversy about the way in which captopril lowers blood pressure. The only known action of captopril is its inhibition of angiotensin converting enzyme—this was seen in our study by the fall in angiotensin II and aldosterone levels. Other studies have directly demonstrated this inhibition¹⁰. If, as seems likely, captopril lowers blood pressure through a fall in angiotensin II, our results indicate that the renin-angiotensin-aldosterone system is a normal mechanism for maintaining blood pressure in

normotensive man. These findings with captopril are supported by the study of Niarchos *et al.*⁸ using teprotide but contrast with those of Haber¹. However both of these studies were short-term infusions in resting subjects.

Inhibition of angiotensin converting enzyme could lead not only to a fall in angiotensin II levels, but also to an accumulation of bradykinin, a potential vasodilator. Recent work has demonstrated that there is no increase in plasma bradykinin with captopril¹⁷ or teprotide¹⁸, although this does not necessarily exclude an increase in tissue bradykinin. Significant correlations between the fall in plasma angiotensin II^{19,20} initial plasma renin activity^{21,22} and the fall in blood pressure with captopril have been shown in patients with high blood pressure and in salt-depleted dogs²³. However, the reduction in the direct vasoconstrictor effect of circulating angiotensin II may not be the only mechanism by which captopril lowers blood pressure²⁴. Angiotensin II has many other actions; by acting on the area postrema in dogs, it can increase blood pressure²⁵. Recent suggestions that the brain renin-angiotensin system may be an important mediator of the response to captopril may also be relevant²⁶. The fall in plasma aldosterone that occurs with captopril could also contribute towards the fall in blood pressure both through the observed loss of sodium and water and by a postulated, but unproved, direct action of aldosterone on arteriolar tone.

Our results also demonstrate that the renin-angiotensin-aldosterone system is an important controller of plasma aldosterone on a normal sodium intake. Either the fall in plasma angiotensin II or aldosterone could account for the observed loss of sodium, so the system is also important in the maintenance of sodium balance on a normal sodium intake.

Finally, if angiotensin II in normotensive human subjects maintains blood pressure on a normal sodium intake, the renin-angiotensin system might be involved in the causation or maintenance of high blood pressure. It would seem likely from our results that, if the levels of angiotensin II in patients with high blood pressure are within the same range as those of normal subjects, the renin-angiotensin system maintains blood pressure to the same extent in hypertensives as in normotensive subjects³.

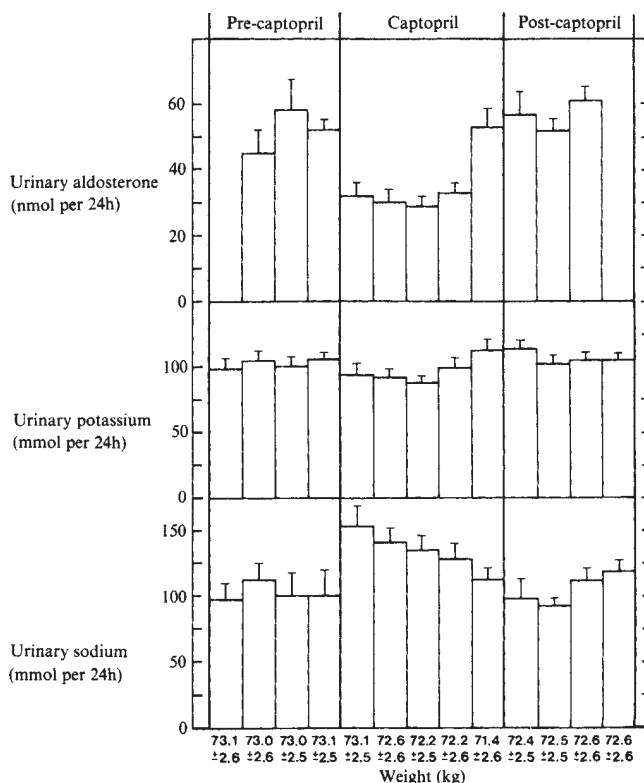


Fig. 3 Mean 24-h urinary aldosterone, potassium, sodium and weight ($\pm$ s.e.m.) in the eight normal subjects for the 4 days before, 5 days of and 4 days after captopril treatment.

and may be an important mechanism whereby blood pressure can be lowered. However, the renin-angiotensin system can only be the cause of high blood pressure if the level of circulating angiotensin II is above normal, relative to the sodium balance, or there is an increased sensitivity to the actions of angiotensin II, compared with that in normotensive subjects.

We thank Professor P. Sever for measurements of plasma noradrenaline. This study was supported by the MRC and the Wellcome Trust.

Received 15 December 1980; accepted 5 March 1981.

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Active immunization and passive transfer of resistance against sporozoite-induced malaria in infant mice

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Immunization attempts using a variety of plasmodial preparations have identified several developmental stages and vaccination procedures which can protect against malaria^{1–3}. These indicate that immunoprophylaxis against malaria may also be possible in man. The immune response to these vaccines has only been investigated in adult animals, but in countries where malaria is prevalent, children are most at risk of developing lethal infections. Thus we must ask whether vaccination against malaria would be effective when applied early in life. Here we report the use of intramuscular (i.m.) immunization of very young mice with radiation-attenuated sporozoites of *Plasmodium berghei* as a vaccination procedure, and that these animals can be effectively protected against sporozoite challenge. This approach avoids the intravenous (i.v.) immunization, which although highly effective in adult animals, is barely acceptable for use in man. Another important aspect of the host-parasite relationship in endemic areas is the role of immunity transmitted congenitally from mother to infants. We therefore investigated whether sporozoite-immunized adult female mice could transfer this immunity to their litters. It was found that the offspring acquired ant sporozoite antibodies from their mothers through the milk. Furthermore, most of these offspring resisted challenge by infective sporozoites.

All experiments were performed in A/J mice using the NK65 strain of *P. berghei*. Infant mice were bred in our animal facility and the adults were obtained commercially (Jackson Laboratories, Bar Harbor). Sporozoites, collected from the salivary glands of laboratory-bred *Anopheles stephensi*, were γ -irradiated at 15,000 rad before being used in immunization⁴.

Young mice, 2–24 days old, and adult mice, 8–10 weeks old, were inoculated i.m. with 7×10^4 irradiated sporozoites suspended in 0.1 ml of tissue culture medium (TC199). These animals received 1–3 additional immunizing doses of 3.5×10^4 irradiated sporozoites, each given by the same route and at intervals of 1–2 weeks. One to two weeks after the last immunizing dose, the immunized animals and age-matched, non-immunized controls were challenged with 1×10^4 infective sporozoites i.v., to test for protection. At the time of challenge, the young mice were 5–7 weeks old. Animals which failed to show parasitized erythrocytes in Giemsa-stained blood smears, 2 weeks after challenge, were considered to be protected.

The results of six experiments, involving the immunization and challenge of 74 young and 24 adult animals, are summarized in Table 1. A high percentage of the immunized young mice developed resistance to sporozoite challenge. The level of protection among the young animals varied from 71 to 100%. The highest per cent protection occurred in those animals which had received the first immunizing dose of sporozoites at the age of 7–14 days. Adult immunized mice had a significantly lower per cent protection. In each of the experiments, the control animals developed parasitaemia within 5 days after sporozoite challenge.

In addition to the demonstration that, in mice, sporozoite vaccination can be effectively administered early in life, our data suggest that the effectiveness of this vaccination varies with the age of the recipient animals. Adult mice are highly protected by i.v. immunization with sporozoites, but when the immunizing sporozoites are given i.m., the per cent protection against challenge decreases considerably⁵. In our experiments, only 33.3% of the adult animals were protected after i.m. immunization (Table 1).

Young animals did not produce detectable serum antibody levels in response to a single dose of irradiated sporozoites. However, after two and three immunizing doses, the young and adult mice had comparable levels of antibodies, as measured by the indirect immunofluorescent antibody test (Table 2).

Further experiments were done to determine whether sporozoite-immunized mice could transfer protection to their offspring; 70 mice corresponding to 13 litters born and nursed by sporozoite-immunized mothers, were challenged with infective sporozoites when the pups were between 18 and 25 days old (Table 3). The challenge had to be delayed until this age because a large proportion of mice <2 weeks old born to and nursed by non-immunized mothers, were found to be resistant to sporozoite-induced *P. berghei* infection (our unpublished

Table 1 Protection against *P. berghei* sporozoite-induced infection of very young and adult mice immunized i.m. with γ -irradiated sporozoites

Age of mice at the start of immunization	Challenged	Protected	Percentage of protection
Infants			
2–6 days	20	16	80.0
7–14 days	44	41	93.2
16–24 days	10	8	80
Adults	24	8	33.3
Non-immunized controls			
Infants	21	0	0
Adults	10	0	0

The data represent the results of six experiments. Irradiated sporozoites were administered 2–4 times, with intervals of 1–2 weeks, each animal receiving a total of 1.05 – 1.75×10^5 irradiated parasites during the immunization period. Non-immunized controls were age-matched with the immunized animals.

Table 2 Serum antibody levels in young and adult animals after i.m. injection of γ -irradiated sporozoites

No. of immunizing doses of sporozoites	log ₂ anti-sporozoite antibody titre	
	Pups	Adult mice
1	<4	5.0 $\pm$ 0.6
2	7.0 $\pm$ 0.5	7.0 $\pm$ 0.3
3	9.2 $\pm$ 0.2	9.0 $\pm$ 0.3

Antibody response as measured by indirect immunofluorescence of young and adult mice to either 1, 2 or 3 i.m. inoculations with irradiated *P. berghei* sporozoites. The primary immunizing dose consisted of 7×10^4 sporozoites; the second and third doses, given at weekly intervals, each consisted of 3.5×10^4 parasites. Serum was collected 2 weeks after completion of each immunization. Anti-sporozoite antibody titres are expressed as mean values $\pm$ s.e.m. for each group of 5 mice. The lowest titre considered as parasite-specific was 4. The experiment was repeated on three different occasions with similar results.

data). Such non-immunological resistance is possibly the result of an exclusive milk diet^{6,7}.

Of the pups born to and nursed by sporozoite-immunized mothers, 60% resisted the infective sporozoite challenge (Table 3). In contrast, age-matched mice born to and nursed by non-immunized mothers were almost uniformly (95%) susceptible to sporozoite challenge. The percentage of resistant offspring of immunized mothers varied from litter to litter. In one experiment, in which we simultaneously challenged four litters using the same sporozoite preparation, the range of protection varied from 28.5 to 100%.

Mice born to and nursed by sporozoite-immunized mothers showed minimal levels of antibodies at birth, which increased gradually with suckling time, reaching maximal levels at 2 weeks old. This progressive increase in antibody level with time suggests that mice acquire antibodies from their mothers largely through the milk. This was further supported by experiments in which mice born to non-immunized mothers were transferred to immune foster mothers. After 12 days of nursing by the latter, the pups had acquired an antibody level similar to that found in age-matched pups born to and nursed by sporozoite-immunized mice. Indirect immunofluorescence antibody titres, obtained at 13 and 21 days of age, were determined for individual serum samples and expressed as a mean value $\pm$ s.e.m. for each group of 5 mice. Titres of the immune mothers were usually a twofold higher dilution than those of the pups. Pups born to non-immunized mothers then transferred to immune foster mothers had log₂ anti-sporozoite antibody titres of 8.6 ± 0.4 and 9.0 ± 0.5 at 13 and 21 days old, respectively, compared with titres of 9.0 ± 0.2 and 9.0 ± 0.3 for pups nursed by immunized mothers. The experiment was repeated three times, with similar results. Protection against sporozoite-induced infection in these animals also seems to be largely transferred through the milk; 55% of the animals born to non-immunized mothers and nursed by sporozoite-immunized mice resisted a sporozoite challenge, whereas all the offspring of immune mice, foster-nursed from

their first day onwards by non-immunized mothers were susceptible to sporozoite infection (data not shown).

The passively acquired antiparasite antibodies largely belong to the IgG class. In contrast, mice actively immunized with sporozoites possess high levels of both IgG and IgM, as detected by immunofluorescence. Whereas the passive transfer of anti-sporozoite antibodies through the milk has been clearly documented in this system, our experiments do not rule out the concomitant transfer of immune cells, which could also contribute to the functional immunity detected in the offspring^{8,9}.

Regarding the role of antibodies in anti-sporozoite-mediated resistance to malaria infection, it has been recently demonstrated that monoclonal antibodies to *P. berghei* sporozoite surface antigen confer complete protection on recipient mice¹⁰. However, our experiments constitute the first instance in which the passive transfer of sporozoite-induced immunity was observed in natural conditions.

In view of these findings in rodents, it is certainly worth investigating whether passive transfer of sporozoite-induced immunity occurs in infant humans living in areas in which malaria is endemic.

We thank Ms Rita Altszuler and Ms Marilyn Maracic for technical assistance, and Ms Sharon Hecht-Ponger for typing the manuscript. A.U.O. is a recipient of a WHO training grant. This work was supported by the WHO and the Agency for International Development.

Received 5 January; accepted 12 March 1981.

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Inducer T lymphocytes synthesize a factor that stimulates proliferation of cloned mast cells

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Inducer T lymphocytes activate other cells to divide and express new function. Known target cells include other lymphocytes and haematopoietic stem cells¹. We now provide evidence that the inducer T cell acts on another important target population: mast cells. Mast cells have a central role in the expression of immediate hypersensitivity and are also prominent in T-cell mediated reactions of the delayed type^{2–7}. Because the proliferation of differentiated cells is often regulated by soluble growth factors, we examined an inducer T-cell clone for its ability to stimulate mast cell proliferation. We report here that cloned Ly1⁺2[–] inducer T cells produce a factor that selectively induces morphologically and karyotypically normal mouse mast cell clones to proliferate. We therefore suggest that inducer T cells may regulate mast cell numbers by releasing a soluble growth factor that stimulates them to divide. Because mast cell products also affect certain T-cell functions^{8–10}, mast cell–T cell interactions may comprise part of an immunoregulatory circuit.

Mast cells proliferate in the thymus of NZB mice¹¹ and elsewhere in association with T cells^{2–7}. To explain these findings, Burnet proposed that certain mast cell populations are

Table 3 Protection against *P. berghei* sporozoite-induced infection of pups born to and nursed by sporozoite-immunized mice

Mothers	Pups			
	No. challenged†	No. protected	Percentage of protection	
No.			Mean	Range
13 Immunized*	70	42	60	28.5–100
7 Non-immunized	22	1	4.5	0–20

The data represent the results of five experiments which included 13 litters of immunized mothers and 7 litters of non-immunized mothers.

*Mice were immunized with a total of 1.1×10^5 γ -irradiated sporozoites i.v., given in five doses before onset of pregnancy.

†Pups were 18–25 days of age when challenged i.m. with 2.1×10^4 infective sporozoites. This inoculum consistently resulted in patent infection in control animals.

Table 1 Newly synthesized histamine content of mast cell or T-cell clones

Clone	Morphology	Newly synthesized histamine (pg per 10^6 cells per 19 h)
Cl. MC/8	Mast cell	4.5×10^4
Cl. MC/9	Mast cell	2.8×10^4
Cl. TL ⁺ Ly1 ⁺ 2 ⁺ /11	Lymphocyte	0
Cl. Ly1 ⁻ 2 ⁻ /11	Lymphocyte	0

Histamine synthesis of two mast cell (Cl. MC/8 and 9) and two T-cell clones was measured by isotopic TLC¹⁶.

derived from cells of the thymus-dependent lineage¹². Alternatively, proliferation might be controlled by a soluble growth factor elaborated by T cells. We evaluated this question further using homogeneous populations of mast cells. Cells derived from the liver of a 13-day old mouse fetus from an A/J female mated with a C57BL/6 male were incubated in media conditioned by concanavalin A (Con A)-activated BALB/c spleen cells¹, and 10 days later were distributed at limiting dilutions in wells containing irradiated (2,000R) syngeneic bone marrow cells. Colonies appeared after 10–14 days with a cloning efficiency of ~10%. No growth of mast cells was observed from preparations of irradiated bone marrow cells. All colonies grown in these conditions were composed of cells of similar appearance that resembled mast cells by light microscopy because of their prominent metachromatic granules and expressed identical profiles of surface membrane glycoproteins (see below). These lines were subsequently cloned by micro-manipulation¹³, and have been designated Cl. MC/1–14. The cloned cells have normal karyotypes and were cultivated in large numbers ($>10^8$) with doubling times of 36–48 h. Clones can be

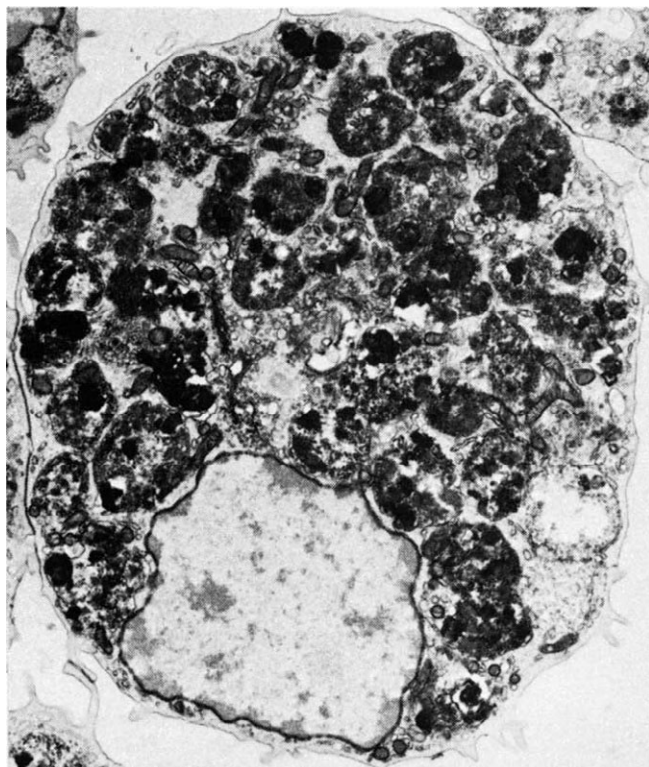


Fig. 1 Electron micrograph of a typical mast cell from Cl. MC/8. Numerous characteristic cytoplasmic granules contain varying amounts of electron-dense particles and small vesicles. By ultra-structural criteria, cloned mast cells closely resemble the mast cells present in murine connective tissue, lymphoid tissue and serous spaces⁵. Cells were fixed and processed by the osmium potassium ferrocyanide method¹⁷. Lead citrate stain, $\times 7,000$.

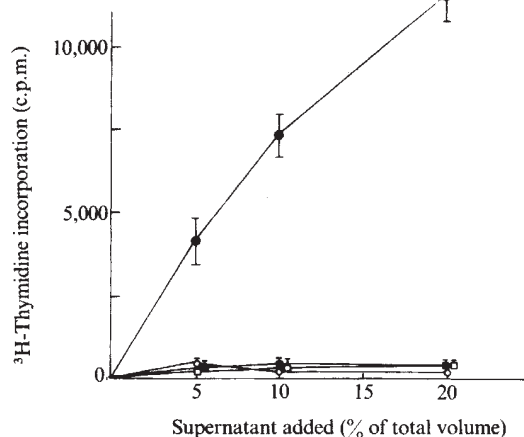


Fig. 2 Proliferation of cloned mast cells in the presence of supernatants of cloned inducer T cells. Cl. MC/9 cells, final concentration of 10^5 cells ml^{-1} , were incubated in flat-bottom microplate wells (Falcon 3040) in 0.1 ml of modified Dulbecco's modified Eagle's medium¹ with 4% heat-inactivated fetal calf serum, 5×10^{-5} M 2-mercaptoethanol and 2 mM glutamine. These cultures were incubated with increasing concentrations of supernatants from different T-cell clones: Cl. Ly1⁺2⁻/9 (●), Cl. Ly1⁺2⁺/4 (○), Cl. Ly1⁺2⁺/16 (■) or Cl. Ly1⁻2⁻/11 (□). After incubation for 24 h at 37 °C in 10% CO_2 , 0.5 μCi of ^3H -thymidine, 5.4 Ci mM^{-1} (NEN), was added. Four hours later, cultures were collected on to glass filter strips and counted by liquid scintillation. Supernatants were prepared as follows: cloned T cells were washed three times and resuspended at a final concentration of 5×10^5 cells ml^{-1} in modified Dulbecco's modified Eagle's media supplemented with 5 $\mu\text{g ml}^{-1}$ human transferrin, 5 $\mu\text{g ml}^{-1}$ bovine insulin, 5×10^{-5} M 2-mercaptoethanol and glutamine (2 mM). After incubation for 24 h at 37 °C in 10% CO_2 , supernatant was collected (300g for 10 min) and filtered (0.22 μm Swinnox).

maintained stably in the absence of any other cell type for at least 5 months, although doubling times may increase. Cl. MC/1–14 cells were further identified as mast cells by electron microscopy (Fig. 1) and by their ability to synthesize and store histamine (Table 1); histamine content ranged from 0.2 to 1.4 pg per cell. Preliminary studies show that cultured mast cells degranulate and release histamine when exposed to non-cytolytic concentrations ($1 \mu\text{g ml}^{-1}$) of the calcium ionophore A23187 or after passive sensitization with IgE and antigenic challenge. In addition, cloned cells can be frozen for storage and thawed with complete recovery of function and phenotype.

Immunofluorescence studies¹ indicate that Cl. MC/1–14 cells do not carry the Thy1, Ly1 and Ly2 surface membrane glycoproteins characteristic of T lymphocytes. However, they do express the Ly5 glycoprotein, as do lymphocytes and haematopoietic cells¹⁴. Mast cells therefore display a different profile of cell-surface glycoproteins from thymocytes or T lymphocytes. We cannot exclude the possibility that thymic lymphocytes and mast cells arise from a common precursor because prothymocytes, like the mast cells described here, express the Thy1⁺Ly5⁺ glycoprotein profile¹⁵. Finally, the ultrastructure and low histamine content of some clones suggest that such clones may represent immature mast cells.

Mast cell clones divide continuously in media conditioned by Con A-activated spleen cells, but not in non-conditioned media. The medium of spleen cells incubated in the absence of Con A does not support growth. To define the cellular source of the mast cell growth factor, conditioned medium was prepared from spleen-derived T-lymphocyte clones¹. Ly1⁺2⁻ inducer T-cell clones, in contrast to other T-cell clones, released a factor into the culture medium which stimulates mast cell proliferation (Fig. 2). The release of this factor is enhanced further by addition of Con A. This material is retained after dialysis, inactivated by pronase and has an apparent molecular weight of 40,000–45,000 on Sephacryl S-200. The partially purified factor does

not stimulate significant proliferation of cloned T cells or fibroblasts (data not shown).

We have shown that pure mast cell populations may be readily and selectively derived from mouse haematopoietic tissue under the influence of an inducer T-cell product. This work is of interest for several reasons. First, such clones should prove useful for investigations requiring purified mast cells, particularly because alternative sources (such as peritoneal cavity mast cells) comprise a heterogeneous population that provides small cell numbers. They may also serve to test whether all mast cells require T-cell signals to the same degree⁷. Second, cloned lines allow definitive characterization of effector and target cell populations. For example, we have identified the mast cell as a direct cellular target of inducer T cells. This effect is not mediated through small numbers of an intervening cell, such as the macrophage, often present in highly enriched T-cell populations. Third, the data provide direct evidence to support earlier observations *in vivo* that link lymphocytes to mast cell hyperplasia in the thymus and at sites of delayed hypersensitivity. It will also be of interest to determine whether inducer T

cells influence the growth of mastocytomas because these mast cell tumours commonly include large numbers of lymphocytes^{6,7}.

Although we have shown that T lymphocytes induce mast cell proliferation, others have demonstrated that mast cell products depress certain T lymphocyte functions. Thus, histamine may inhibit *in vitro* generation of cytotoxic T lymphocytes⁸, macrophage inhibitory factor production⁹, and cytotoxic T-lymphocyte effector function¹⁰. Taken together, these findings point to mutual interactive relationships between T lymphocytes and mast cells. These cellular interactions may explain mast cell hyperplasia in a variety of clinical and experimental hypersensitivity settings and suggest a control mechanism to curtail such reactions.

We thank Justine Osage and Rachel Pocinki for technical assistance. This work was supported by USPHS grants GM 07753, AI 12184, AI 13600, CA 28834 and AI 16925.

Note added in proof: Cl.Ly1⁺2⁻ mast cell growth factor has been purified to apparent homogeneity. It has a pI of ~6.0 and η of 45,000 (refs 1, 18).

Received 27 October 1980; accepted 10 March 1981.

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In vivo effect of anti-asialo GM1 antibody on natural killer activity

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Mounting evidence suggests that natural killer cells (NK) play an important part in the destruction of incipient tumours. If that is so, the destruction of NK cells *in vivo* should allow tumour cells to proliferate. We have recently described an antiserum having anti-NK activity which was raised against mouse brain tissue, and its effective antibody was shown to be directed against the cell surface glycolipid GM1 with its sialic acid groups removed (asialo GM1)¹. We show here that intravenous (i.v.) injection of microlitre amounts of anti-asialo GM1 into BALB/c nu/nu mice almost completely abolishes NK activity and enhances the local growth of syngeneic lymphoma, RL δ 1 as well as the incidence of tumour take. Evidence suggests that this effect is due to membrane damage producing loss of NK function rather than to cytolytic membrane attack.

The anti-asialo GM1 used in this study was prepared as an ammonium sulphate precipitate of whole antiserum of rabbits immunized with asialo GM1 as described previously¹. As shown in Fig. 1, *in vivo* administration of anti-asialo GM1 to BALB/c nude mice resulted in a marked reduction in NK activity. The amount of anti-asialo GM1 required for elimination of NK activity was much smaller than expected. The dose response curve clearly indicates that even a few microlitres of anti-asialo GM1 is enough to eliminate almost all the NK activity in nude mouse spleen cells. Similar results were also obtained in various

lymphoid organs (data not shown). The repressed state of NK activity lasted for several days, up to 1 week after one injection of the antiserum. Thus, we thought that anti-asialo GM1 could be used to study the biological role of NK cells *in vivo*. Table 1 shows that i.v. injection of anti-asialo GM1 into BALB/c nude mice greatly enhanced the local growth of syngeneic lymphoma, RL δ 1 as well as the incidence of tumour take. This strong association between NK function and resistance to tumour growth agrees with the previous result which indicates the retardation of tumour growth by positively selected NK cells². There are also remarkable parallels between the present results

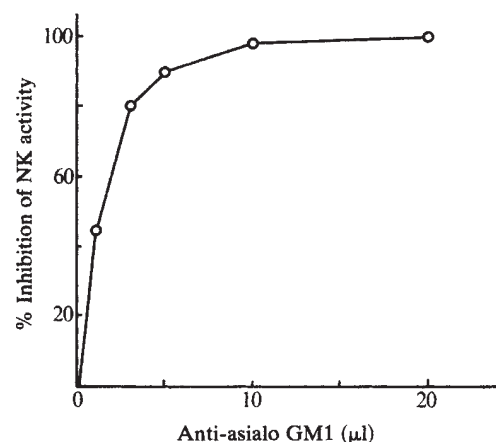


Fig. 1 *In vivo* effect of anti-asialo GM1 on NK activity. BALB/c nu/nu mice were injected i.v. with 0.1 ml of serially diluted anti-asialo GM1 and NK activity of spleen cells was determined after 24 h. The immunization protocol and determination of specificity for anti-asialo GM1 antiserum are described elsewhere¹. NK activity was determined as follows: 2×10^4 ⁵¹Cr-labelled YAC-1 cells (a Molony virus-induced lymphoma in A/Sn mice) were incubated in RPMI 1640 medium plus 10% fetal calf serum with (1×10^6) spleen cells at 37 °C in 5% CO₂. The amount of γ radioactivity released from triplicate cultures after 4 h was determined.

Table 1 Effect of anti-asialo GM1 on tumour takes in BALB/c nude mice

Injected with	Frequency of tumour takes*					
	Days after inoculation:					
	0	4	8	16	20	24
NRS	0/20	0/20	3/20	5/20	5/20	6/20
Anti-asialo GM1	0/20	16/20	16/20	20/20	20/20	20/20

BALB/c *nu/nu* mice were injected i.v. with 0.1 ml of anti-asialo GM1 (diluted 1:10) and then 2×10^6 RL δ 1 cells were inoculated subcutaneously. The same amount of anti-asialo GM1 was injected repeatedly at intervals of 3 days. As a control, the same amount of normal rabbit serum (NRS) instead of anti-asialo GM1 was injected.

* No. of animals showing tumour growth out of the total no. grafted.

and the recent observation indicating that the growth of transplanted tumour cells is enhanced in beige mice with impairment of NK activity^{3,4}. Furthermore, subsequent studies have shown that anti-asialo GM1 could be of practical value in improving the transplantation of human tumour cells which do not readily take even in nude mice⁵.

As the reduction of NK activity was also observed even 1 h after injection of anti-asialo GM1, we set up an experiment to determine the mode of action of anti-asialo GM1 on NK activity. As can be seen in Table 2, the incubation of BALB/c *nu/nu* spleen cells with anti-asialo GM1 for 1 h at 37°C resulted in a strong reduction in NK activity only when normal mouse serum (NMS) was present. As was the case *in vivo*, NK activity was inhibited by even a very small amount of anti-asialo GM1, whereas NMS alone was not effective. Because of the weak activity of mouse complement, it seems unlikely that abolition of NK activity is due to the cytolytic effect caused by microlitre amounts of anti-asialo GM1. In fact, reduction of NK activity was caused by serum from AKR mice with a genetically determined deficiency of C5 as well as by serum from mouse strains possessing the complete spectrum of complement components (Table 2). Serum from C4-deficient guinea pigs was also effective in removing NK activity in the presence of anti-asialo GM1. These results indicate that the cytolytic membrane attack pathway is not required for this reaction. It seems reasonable to assume that anti-asialo GM1 antibody and heat-labile factor(s) in NMS cause a certain membrane damage which gives rise to loss of NK function, rather than to the cytolytic membrane attack pathway.

In any event, the distinct *in vivo* effect of anti-asialo GM1 in nude mice can probably help us to understand some aspects of natural defense mechanism against neoplastic growth. Furthermore, it is quite conceivable that a very small amount of

Table 2 Requirement of serum factor for elimination of NK activity by anti-asialo GM1 *in vitro*

Experiment	Treatment of BALB/c <i>nu/nu</i> spleen cells	NK activity (% lysis)
a	Anti-asialo GM1 alone	32.1 $\pm$ 1.5
	NMS (BALB/c <i>nu/nu</i>) alone	31.4 $\pm$ 1.1
	NMS + anti-asialo GM1	0.5 $\pm$ 0.3
	NMS (56°C, 30 min) + anti-asialo GM1	30.7 $\pm$ 1.2
b	NMS (AKR) alone	31.2 $\pm$ 1.5
	NMS + anti-asialo GM1	0.8 $\pm$ 0.4
c	GPC (C4-deficient guinea pig serum) alone	30.0 $\pm$ 0.9
	GPC + anti-asialo GM1	1.1 $\pm$ 0.8

NK activity of BALB/c *nu/nu* spleen cells was determined after the following treatments. *a*, Spleen cells (5×10^6 per 0.1 ml) were incubated for 1 h at 37°C with anti-asialo GM1 (1:100) diluted in the serum of BALB/c *nu/nu* mice. *b*, Spleen cells were incubated for 1 h at 37°C with anti-asialo GM1 (1:100) diluted in the serum of AKR mice. *c*, Spleen cells were incubated for 1 h at 37°C with anti-asialo GM1 (1:100) diluted in C4-deficient guinea pig complement (GPC). Effector/target ratio was 50:1.

autoantibody to asialo GM1 could be the key immunoregulatory negative feedback signal for NK activity.

We thank Dr H. Okada for C4-deficient guinea pig serum and Ms N. Katsu for secretarial help.

Received 11 November 1980; accepted 9 March 1981.

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Interleukin-2 augments natural killer cell activity

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The lymphokine interleukin-2 (IL-2; also termed T-cell growth factor) causes the proliferation of activated T-cell clones^{1,2}. Effector T-cell lines exhibiting suppressor, helper and cytotoxic activities have been established by growth of appropriately stimulated cells in IL-2-containing medium^{3,4}. In addition, IL-2 has been shown to provide requisite T-cell 'help' in a number of *in vitro* immune response assays, including the generation of cytotoxic T cells from thymocyte (and nude spleen cell) cultures⁵ and the induction of erythrocyte-specific antibody in T cell-deficient populations⁶. We report here a further activity of IL-2, one previously attributed solely to interferon—the ability to augment the cytotoxic activity of natural killer (NK) cells. This conclusion was reached from the observation that preparations of IL-2, which lacked interferon, caused a rapid increase in NK cell activity. This ability to augment cytotoxicity was removed from IL-2 preparations, not only by absorption with IL-2 receptor bearing cells, but also by precipitation with a monoclonal antibody directed against IL-2.

Our attention was originally drawn to IL-2 as an NK cell potentiator when we observed that spleen cells from adult C57BL/6, CBA/J and BALB/c nude (*nu⁺/nu⁺*) mice showed markedly increased NK cell activity when cultured for short (up to 24 h) periods in medium containing IL-2. As previously reported⁷, murine NK cell reactivity was relatively labile in conventional culture conditions, and decreased ~60–80% after culture for 24 h. In contrast to this decline, cells cultured in medium containing IL-2 showed markedly increased NK cell reactivity, even when compared to the activity of fresh uncultured spleen cell populations. Increases in splenocyte NK cell reactivity were first observed after ~16 h of culture in IL-2-containing medium. The level of augmentation observed peaked after 24–30 h of culture and declined to startling levels by ~96 h.

Analysis of a variety of IL-2 preparations for conventional IL-2 activity (ability to support the proliferation of an IL-2-dependent cell line, as measured in a 24-h microassay⁸ and concurrently for NK augmenting activity (ability to augment NK cell activity during a 24-h culture period; and subsequently assessed in a 4-h ⁵¹Cr release assay⁹), showed a positive correlation between the two activities (Table 1).

It has been widely observed that interferon augments NK-cell reactivity^{10,11}, but several observations led us to believe that the augmentation caused by the IL-2 preparations was not due to contaminating interferon. First, in those experiments in which IL-2 preparations were added to graded doses of a purified mouse interferon preparation, augmentation of NK-cell reactivity was observed over and above that elicited by interferon alone (Fig. 1). In the conditions used, 10^3 units ml⁻¹ interferon had maximal stimulatory effects, with amounts in excess of this

Table 1 Effect of IL-2 containing preparations on murine NK-cell activity

Effector cells	Culture conditions	% Specific cytotoxicity against:			
		IL-2 (U ml ⁻¹)	YAC-1	L5178Y cl 27v	L5178Y cl 27av
BALB/c (nu ⁺ /nu ⁺) spleen	Not cultured	—	21.7	14.7	1.1
	Medium alone	0	6.5	1.9	0.9
	Rat IL-2 ^a	2.2	60.0	40.6	5.8
	PAGE-purified mouse IL-2	3.5	72.0	ND	ND
	Medium alone	0	3.3	0.1	0.8
	Rat IL-2 ^b	0.6	24.2	12.7	1.3
	Syngeneic MLC supernatant (C57BL/6 + C57BL/6Mit)	0.05	9.6	5.2	2.9
	Congenic MLC supernatant (B10.BR + AKR/JMit)	1.5	38.9	20.4	5.1
	Allogeneic MLC supernatant (C57BL/6 + CBA/JMit)	2.4	50.5	27.7	7.6
	Medium alone	0	1.5	ND	ND
	Isoelectrically focused IL-2: pI 4.3	1.7	23.6	ND	ND
	pI 4.9	1.5	22.7	ND	ND

10⁷ spleen cells were cultured for 24 h at 37 °C in 2 ml of RPMI 1640 medium containing 10% fetal calf serum and 5 × 10⁻⁵ M 2-mercaptoethanol. At the end of culture, the cytotoxic activity was assessed in a 4-h ⁵¹Cr-release assay with YAC-1, cl 27v and cl 27av as target cells. A range of effector:target cell ratios was used; the data shown are for multiplicities of 50:1. Cl 27v and cl 27av are both cell lines established from the DBA/2 lymphoma L5178Y which differ in their susceptibility to NK cells, cl 27v being susceptible, cl 27av being insensitive²⁰. Both of these lines are lysed to an equivalent extent by alloimmune cytotoxic T cells²⁰. The cytotoxic activity measured above was attributed to NK cells by a number of criteria, but notably by the ability to lyse 27v cells but not 27av, and by the observation that the lytic activity was abrogated by pretreatment of effector cells with complement and an antiserum directed against the neutral glycolipid, asialo GM₁ which is displayed on NK cells but not on cytotoxic T lymphocytes²¹. The IL-2 preparations used were: rat IL-2 supernatants from 24 h Con A (5 µg ml⁻¹) stimulated rat spleen cells (10⁷ cells ml⁻¹) used either at a dilution of 1:20 or diluted 1:80; purified mouse IL-2 supernatant from PHA-stimulated LBRM lymphoma cells¹⁴ eluted off a gel slice from a polyacrylamide gel (PAGE) or from an isoelectric focusing gel, as indicated (for experimental details of preparations see refs 13, 14); murine MLC supernatants (diluted 1:2) from 24-h culture of 10⁷ responder spleen cells and 10⁷ mitomycin-C treated congenic or allogenic splenocytes as indicated. ND, not determined. Similar findings to those shown were also made using CBA/J spleen cells as a source of NK cells.

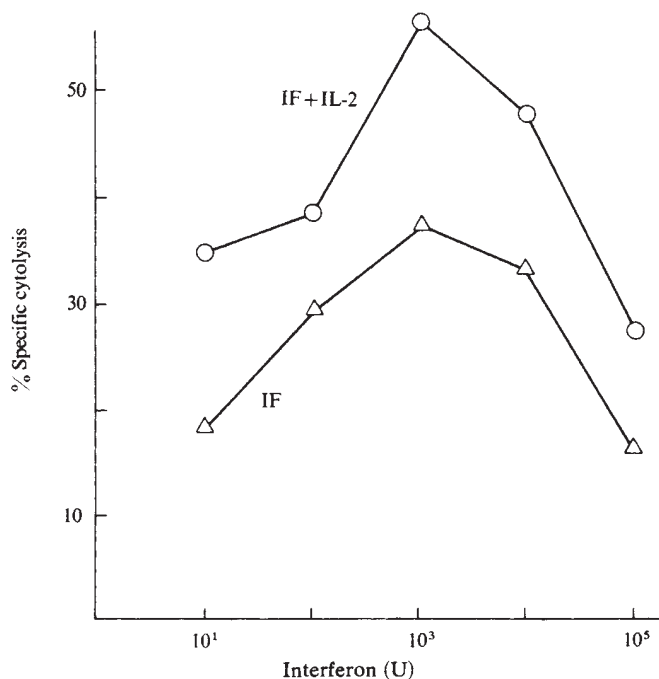


Fig. 1 Effects of interferon (IF) and interleukin-2 (IL-2) on the activity of CBA/J splenic NK cells. Various amounts of purified mouse fibroblast IF (1.1 × 10⁷ units per mg protein, Calbiochem-Behring) were added to 10⁷ normal CBA/J spleen cells and incubated for 24 h at 37 °C in the presence or absence of 1 unit of purified murine IL-2 (prepared by sequential (NH₄)₂SO₄ precipitation, ion-exchange chromatography and flat bed isoelectric focusing of mitogen-induced mouse spleen cell conditioned medium using methods described elsewhere¹⁶). After 24 h, the NK-cell mediated lytic activity of the cultures was assessed by a 4-h ⁵¹Cr release assay using YAC-1, L5178Y cl 27v and 27av target cells, at various effector:target cell ratios. The data shown are for YAC-1 target cells and an effector:target cell ratio of 25:1. △, Interferon alone; ○, interferon plus IL-2.

causing less stimulation. Nevertheless, even at those doses, the addition of IL-2 was associated with increased NK cell-mediated cytotoxicity, clearly implying that at least some of the potentiating effects of IL-2 differed from, and were additive to, those induced by interferon. This conclusion was supported by observations that several IL-2 preparations which had no demonstrable interferon activity, induced considerable augmentation of the cytotoxic activity of NK cells.

Table 1 illustrates the NK-cell potentiating effects of several IL-2 preparations as tested on BALB/c (nu⁺/nu⁺) responder spleen cells. Similar results were also found for CBA/J splenocytes. Concanavalin A (Con A)-induced rat IL-2 caused significant augmentation of murine NK cell activity, as did supernatants obtained from murine mixed lymphocyte cultures (MLCs). It is important to note that NK-cell potentiation was obtained using supernatants collected from a number of MLC combinations, including those (M locus differences) which do not elicit interferon production¹². In agreement with the notion that IL-2 can potentiate NK-cell reactivity, the most potent augmentation of cytotoxicity was obtained with a purified IL-2 preparation from phytohaemagglutinin (PHA)-stimulated LBRM-33 murine lymphoma cells and purified by successive gel filtration, ion-exchange chromatography, preparative flat-bed isoelectric focusing and polyacrylamide gel electrophoresis (referred to in Table 1 as PAGE-purified IL-2; see ref. 13). IL-2 prepared from mitogen-stimulated LBRM cells by isoelectric focusing¹⁴ was also active in potentiating NK-cell reactivity. Neither the isoelectrically purified IL-2 preparations nor those isolated by polyacrylamide gel electrophoresis showed interferon activity as assayed by virus neutralization¹⁵ (M. Proffitt, personal communication). These findings strongly suggested that IL-2 was capable of augmenting NK cell-mediated cytotoxicity. Such conclusions were strengthened by studies in which IL-2 activity was specifically removed from the preparations.

A number of cells behave, at least operationally, as though they bear receptors for IL-2 (ref. 16). They remove conventional IL-2 activity from media containing the lymphokine in short-term adsorption trials (at 4 or 37 °C), regardless of whether the

Table 2 Loss of ability of IL-2 preparations to augment NK-cell reactivity following adsorption with Con A 'blasts'

Source of IL-2	Adsorption of IL-2 with:	IL-2 activity U ml ⁻¹	NK cell mediated lysis
None	—	0	7.5
Rat spleen	None	1.25	36.8
	Normal spleen	1.10	30.6
	Con A 'blasts'	<0.05	5.4
None	—	—	12.3
Mouse MLC	None	2.3	32.5
	Normal spleen	2.1	33.8
	Con A 'blasts'	0.25	16.6

The NK-cell reactivity of CBA/J spleen cells was assessed after culture (16 h 37 °C) with or without, the IL-2 preparations noted. NK-cell mediated lysis was assessed in a 4-h ⁵¹Cr release assay using YAC-1 target cells. The data shown were obtained at an effector: target cell multiplicity of 50:1. The IL-2 preparations used were obtained either from culture supernatants following Con-A stimulation of rat spleen (5 µg ml⁻¹, 24 h) or by collecting supernatants from 24-h MLC cultures between 10⁷ B10.BR and 10⁷ MLC-treated AKR spleen cells. In the case of mitogen-induced IL-2, a 1:40 dilution (1.25 U) was used, in the case of the MLC supernatant a 1:2 dilution (2.3 U) was used. The adsorption procedure was as follows: 100 µl of IL-2 containing medium was incubated (4 °C, 4 h) with (1) 10⁷ normal C57BL/6 spleen cells or with (2) 10⁷ C57BL/6 spleen cells previously stimulated for 24 h with 5 µg ml⁻¹ Con A. At the end of incubation, the cells were removed by centrifugation and the supernatants filter-sterilized and tested for both NK augmenting activity and for conventional IL-2 activity.

adsorbing cell population is living, or fixed with glutaraldehyde¹⁶. Cells exhibiting this property include all IL-2-dependent effector T-cell lines thus far analysed, as well as mitogen-activated T-cell blasts¹⁷. Unstimulated lymphoid cells do not adsorb conventional IL-2 activity and thus apparently lack, or express very few, IL-2 receptors¹⁶. Culture supernatants from MLC-stimulated murine spleen cells, or Con A-stimulated rat spleen cells, were tested for their capacity to potentiate NK-cell reactivity both before and after adsorption (4 h, 4 °C) with either unstimulated or mitogen-activated T-cell populations (10⁸ cells per ml). As can be seen in Table 2, the ability of the supernatants to potentiate the cytotoxic activity of NK cells was unaffected after adsorption with normal spleen cells. Similarly, adsorption with resting spleen cells had no effect on the ability of the supernatant to induce the proliferation of an effector T-cell line (measured as U ml⁻¹ IL-2 activity). The NK potentiating

activity, as well as its conventional IL-2 activity, was however totally removed when Con A-activated splenocytes were used for adsorption. Both measurable T-cell proliferation and NK potentiation activities were similarly removed from the supernatants after adsorption with either of two long-term IL-2-dependent T-cell lines (data not shown).

We have recently described the preparation of a murine hybridoma which secretes an IgG antibody capable of inhibiting IL-2 activity¹⁸. This hybridoma was obtained by the fusion of spleen cells from BALB/c mice, previously immunized with rat IL-2, with the BALB/c myeloma SP2, according to the procedure of Kohler and Milstein¹⁹. We have isolated three hybridoma clones which secreted antibody that was able to neutralize (or precipitate, together with an additional complexing reagent) rat, mouse or human IL-2 activity. In tests of the effects of antibody with this specificity on the augmentation of NK-cell activity by IL-2 preparations (Table 3), although a small amount of NK-cell potentiating activity was lost nonspecifically by Protein-A bead mixing, only the anti-IL-2 antibody preparation was able to abrogate the NK-potentiating activity of the IL-2 preparations. This implies that the lymphokine IL-2 was, in fact, responsible for the augmentation of cytotoxicity by the lymphokine preparation.

There has been much recent interest in the ability of interferon (and of interferon-inducing agents) to augment NK-cell reactivity both *in vivo* and *in vitro*. Indeed, such observations seem to form the conceptual basis from which widespread clinical trials to assess the anti-neoplastic effects of interferon, have been launched. Thus far, the NK-augmenting effects of such agents as BCG, *Corynebacterium parvum* and poly I:C has been attributed exclusively to interferon. Although this may well be the case, this report indicates that interferon is not alone in its ability to promote NK cell-mediated cytotoxicity, leading one to question whether some of the potentiating affects previously attributed to interferon might be related not to this lymphokine, but to interleukin-2. In any case, our study suggests that IL-2, which has previously been shown to regulate a wide variety of immune responses, might be studied more closely as a potential immune response modifier, particularly in those situations in which NK cells may play a salient role.

This work was supported by NIH grants AI 15384, CA 24537 and CA 28419, grant IM-274 from the American Cancer Society and by a grant from the National Foundation. S.G. is a Special Fellow of the Leukemia Society of America. We thank Dr M. Proffitt for carrying out interferon assays on purified IL-2 preparations.

Table 3 Effect of anti-IL-2 monoclonal antibody on the potentiation of NK cell reactivity by IL-2 preparations

Effector Cell	IL-2 preparation from:	Antibody added	Passage through Protein-A-Sephadex column	IL-2 remaining after treatment (U ml ⁻¹)		NK-cell mediated lysis	
				Expt 1	Expt 2	Expt 1	Expt 2
BALB/c (nu ⁺ /nu ⁺)	None	—	—	—	—	8.2	1.5
	Rat spleen	—	—	1.9	1.8	34.9	22.0
		—	+	1.5	1.7	24.0	18.9
		Anti-IL-2	+	0.2	<0.05	8.6	1.6
		Anti-gp70	+	ND	1.7	ND	18.1
CBA/J	None	—	—	—	—	10.0	7.6
	Rat spleen	—	—	3.8	1.9	38.5	22.8
		—	+	3.0	1.7	24.7	15.3
		Anti-IL-2	+	0.4	<0.05	6.8	1.0
		Anti-gp70	+	ND	1.6	ND	20.7

Cell-free supernatants obtained from Con A-stimulated rat splenocytes or 24-h murine MLC cultures (Table 1) were incubated with an equal volume of a monoclonal IgG antibody of indicated specificity (or with plain medium) for 30 min at 37 °C. These mixtures were then further incubated (or not) with protein A-conjugated Sephadex beads in an end-over-end shaker for 2 h at room temperature. The beads were then removed by centrifugation and the treated supernatants assessed for their ability to augment NK mediated cytotoxicity (of YAC-1 targets after culture for 16 h with BALB/c (nu⁺/nu⁺) or CBA/J spleen cells in a 4-h ⁵¹Cr release assay) and concomitantly for conventional IL-2 activity (as measured (U ml⁻¹) by proliferation of an IL-2 dependent cell line). Con A (0.3 µg ml⁻¹) and supernatants from 24-h syngeneic MLC cultures were tested similarly at the same time for both NK augmenting activity and for conventional IL-2 activity and were found to give only background levels of each activity (data not shown).

Received 12 December 1980; accepted 10 March 1981.

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Synthesis and secretion of mouse immunoglobulin chains from *Xenopus* oocytes

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The secretion of immunoglobulins from mouse plasmacytoma cell lines is a valuable model for the study of mechanisms of protein secretion from eukaryotic cells. Some cells do not synthesize heavy chain immunoglobulin while retaining the ability to synthesize though not to secrete the complementary light chain¹⁻³. In the MOPC 21 variant line, NS1, it is absence of heavy chain that prevents secretion⁴⁻⁶. A reciprocal dependence, that is, a requirement of light chain for heavy chain secretion, has not been tested directly because no variants producing normal heavy chain alone have been detected^{4,7-9}. In view of the known insolubility characteristics of isolated heavy chains *in vitro*¹⁰⁻¹², this failure has led to the idea that free heavy chains would be toxic to the cell^{7,9}. Recently, *Xenopus laevis* oocytes have been shown to provide a surrogate system for studying protein secretion after microinjection of the encoding mRNA^{13,14}. Using this technique we demonstrate here that tetrameric mouse immunoglobulin is assembled and secreted by the oocyte but that free light chains are not secreted. Moreover, using purified heavy chain mRNA we show that heavy chain dimers accumulate in the oocyte and are not secreted unless light chain mRNA is also injected.

The cell line P3/X63 produces an immunoglobulin (MOPC 21) consisting of $\gamma 1$ heavy chains and κ light chains¹⁵. When mRNA from this cell line is injected into oocytes both heavy (H) and light (L) chain proteins are synthesized, sequestered within membranes and secreted (Fig. 1, tracks 1-3); these chains co-migrate with the analogous products prepared from the X63 cells and media (Fig. 1, tracks 4, 5). However, comparison of the ³⁵S-methionine contents of secreted and intracellular heavy and light chains reveals that a disproportionate amount of heavy chain is secreted by oocytes, though not by cultured cells. In fact, when allowance is made for the different numbers of methionine residues in each chain (four in light chain, nine in heavy chain^{16,17}), it is evident that whereas approximately equal amounts of each chain are secreted, excess light chain accumulates within the oocytes. As only assembled (tetrameric H₂L₂) IgG is secreted from the X63 cells⁵, these results suggest that

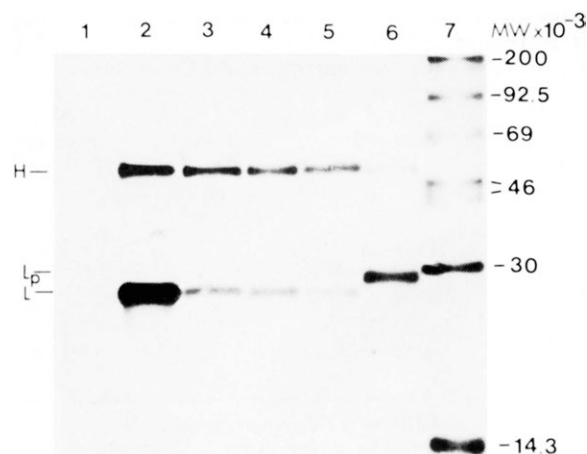


Fig. 1 Synthesis and secretion of X63 immunoglobulin. Culture and labeling of plasmacytoma cells: P3/X63, P3/NS1 and PuBu1 cells (supplied by D. Secher) were grown in Dulbecco's modified Eagle's medium containing 10% horse serum (Difco). Cells were labelled with ³⁵S-methionine (Radiochemical Centre) as described by Cotton *et al.*². Media and cell homogenates were retained for immunoprecipitation and electrophoresis. mRNA preparation: 5×10^9 cells were lysed in hypotonic buffer²¹ containing 0.1% diethylpyrocarbonate (Sigma). The post-nuclear supernatant was extracted with phenol and then precipitated with ethanol. The RNA pellet was resuspended in 5 ml of 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1% SDS and 0.5 M LiCl, bound to oligo(dT)-cellulose (type III, Collaborative Research) and eluted with the same buffer minus LiCl. The polyadenylated mRNA was then ethanol precipitated and resuspended in distilled water at 1 mg ml⁻¹. Microinjection and culture of oocytes: oocytes were microinjected with 30 nl of mRNA and cultured in Barths saline (5 oocytes per 30 μ l) containing 1 μ Ci ml⁻¹ ³⁵S-methionine (300 Ci mmol⁻¹) for 24 h at 21 °C (ref. 13). Culture media were retained for electrophoresis and oocytes were fractionated on sucrose step gradients as previously described¹³. Essentially two fractions were obtained: a vesicle fraction derived from oocyte intracellular membranes and a cytosol fraction containing the soluble components of the oocyte. Immunoprecipitation and electrophoresis: 50- μ l aliquots of cell lysates, oocyte fractions and culture media were immunoprecipitated with 3 μ l of goat anti-mouse IgG (Miles) using *Staphylococcus aureus* envelopes as described by Curtis *et al.*²², the only modification being the use of immunoprecipitation buffer containing 1% Triton X-100 (v/v), 1% sodium deoxycholate (w/v), 0.5% SDS (w/v), 1 mM phenylmethylsulphonyl fluoride, 100 mM KCl, 5 mM MgCl₂ and 100 mM Tris-HCl, pH 8.2 (A. Williamson, personal communications). Precipitates were dissolved in sample buffer (20 mM Tris-HCl pH 7.6, 20% glycerol, 1 mM phenylmethylsulphonyl fluoride, 1% β -mercaptoethanol, 0.01% bromophenol blue and 2% SDS, and 25- μ l aliquots run on 12.5% polyacrylamide gels²³, followed by fluorography²⁴. Track 1, oocyte cytosol; 2, oocyte vesicle; 3, oocyte medium; 4, X63 cell lysate; 5, X63 cell medium; 6, X63 mRNA translated in reticulocyte lysate¹⁵; 7, molecular weight (MW) markers (Radiochemical Centre). Abbreviations: H, heavy chain; L, light chain; L_p, light chain produced in reticulocyte lysate.

only assembled IgG is secreted by the oocyte. This possibility was examined by analysing the intra- and extracellular immunoglobulin on non-reducing polyacrylamide gels. As can be seen in Fig. 2a (track 2) two major species are present intracellularly in the oocyte, corresponding to the tetrameric IgG and free light chains. Surprisingly, very little tetramer can be seen in the oocyte medium (Fig. 2a, track 3), in contrast to the X63 medium (track 5). Instead, trimers, dimers and monomers are observed. However, when 6% dialysed horse serum is included in the oocyte medium (Fig. 2b), most of the secreted IgG is in the tetrameric form (compare tracks 2 and 4); presumably, the added serum reduces gratuitous oxidation of the external immunoglobulin.

A further demonstration of the fidelity of the oocyte in its surrogate role is seen after microinjection of the mRNAs encoding the κ light chains of the cell lines NS1 and P1. Of these two light chains only the P1 κ -chain is secreted *in vivo* both in an assembled form (with the P1 $\gamma 2a$ heavy chain) and free⁵. When P1 and NS1 mRNAs are injected separately or together, it is clear that only the P1 κ -chain is secreted even when both κ -chains co-exist in the same oocyte (Fig. 3). Although a rescue of the NS1 κ -chain by assembly with the P1 $\gamma 2a$ heavy chain might have been anticipated, in view of a similar phenomenon in P1/NS1 hybrids⁵, very little $\gamma 2a$ heavy chain was synthesized in

Fig. 2 Assembly of immunoglobulins in oocytes. Oocytes were injected with X63 mRNA, NS1 mRNA or X63 heavy chain mRNA (see Fig. 4a legend), cultured and fractionated as described in Fig. 1 legend. Aliquots (50- μ l) were then alkylated with iodoacetic acid before immunoprecipitation. Precipitates were eluted from *S. aureus* in 50 μ l of sample buffer without β -mercaptoethanol, boiled at 100 °C for 2 min and divided into two 25- μ l aliquots. One aliquot (non-reduced) was ready for use, the second was reduced and completely alkylated²⁵ before electrophoresis on exponential 7.5–20% gradient gels as described in Fig. 1 legend; a–c illustrate independent experiments. a, Track 1, oocyte vesicles, NS1 mRNA; 2, oocyte vesicles, X63 mRNA; 3, oocyte medium, X63 mRNA; 4, X63 cell lysate; 5, X63 cell medium. b, Track 1, oocyte vesicles, X63 mRNA; 2, oocyte medium, X63 mRNA; 3 and 4, as tracks 1 and 2, respectively, except that incubation medium contained 6% dialysed horse serum. c, Track 1, oocyte vesicles, heavy chain mRNA; 2, oocyte medium, X63 mRNA; 3, oocyte vesicles, X63 mRNA; 4, as track 1.

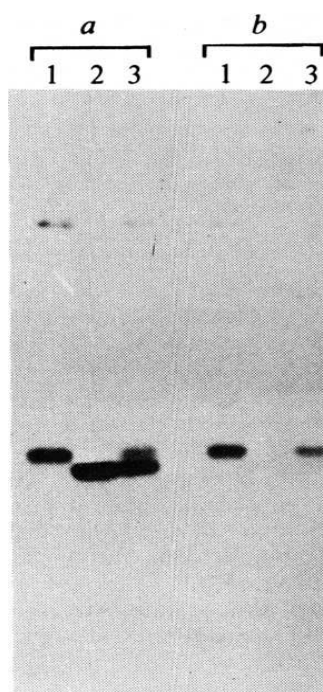
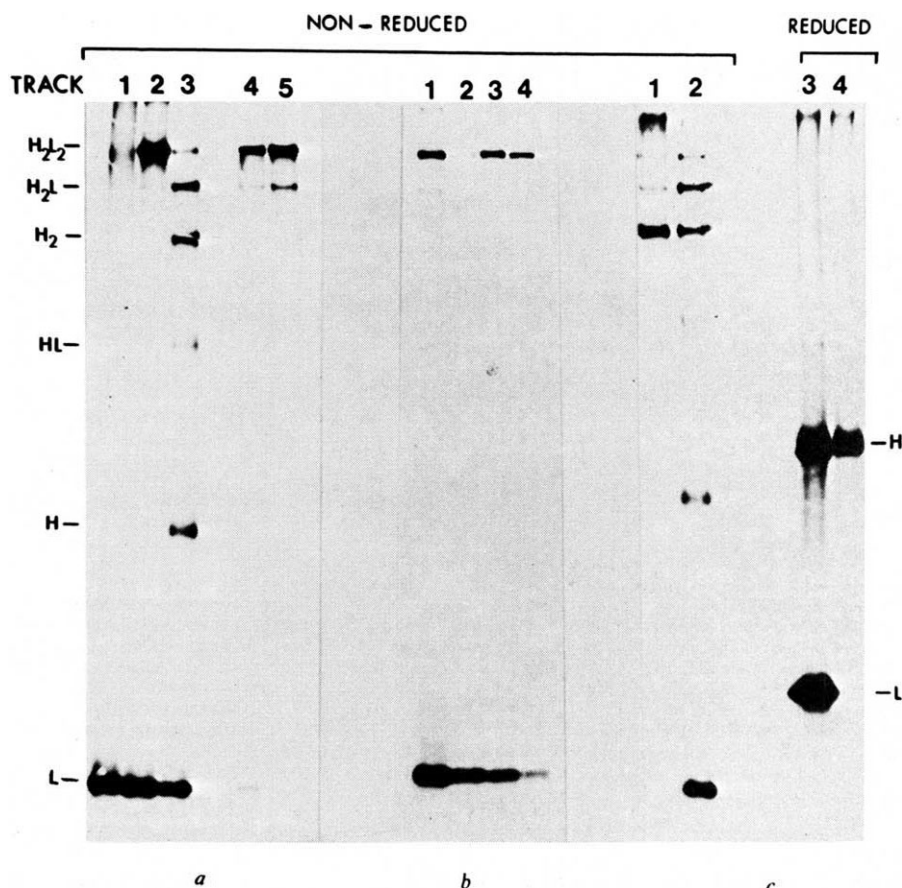


Fig. 3 Synthesis and secretion of NS1 and P1 immunoglobulins. Oocytes were microinjected with NS1 or P1 mRNAs or NS1 plus P1 mRNA and incubated in ³⁵S-methionine as described in Fig. 1 legend. Oocytes were then homogenized in phosphate-buffered saline (40 μ l per oocyte) containing 1% Triton X-100 and 1 mM phenylmethylsulphonyl fluoride and then clarified by centrifugation. Oocyte homogenate and incubation media were immunoprecipitated, alkylated and then electrophoresed on 12.5% gels. In terms of amount per oocyte, six times more sample was electrophoresed in the oocyte media tracks than the corresponding homogenate tracks. Homogenates or media were prepared from oocyte injected with P1 mRNA (1), NS1 mRNA (2), NS1 + P1 mRNA (3). a, Oocyte homogenate; b, oocyte incubation media. H, heavy chain; L, light chain.

these experiments. As the NS1 and X63 light chains are identical in amino acid sequences (C. Milstein, personal communication), these experiments demonstrate that the NS1 light chain cannot be secreted from oocytes in the absence of heavy chain (compare Fig. 3, tracks 2, with Fig. 1, tracks 2, 3).

As mentioned earlier, cell lines lacking light chain but synthesizing normal γ heavy chain have not been detected. We have tested the intrinsic secretory potential of isolated heavy chains by separating heavy and light chain mRNAs on formamide-agarose gels (Fig. 4a) and then injecting the re-

covered mRNAs both separately and recombined into oocytes. As can be seen in Fig. 4b, heavy chain alone is synthesized and sequestered within oocyte membranes but it is not secreted unless purified light chain mRNA is also injected. This rescue is also affected by co-injection of either NS1 mRNA (Fig. 4b) or the MOPC 315 λ II light chain mRNA (A. Colman, C. Valle, T. R. Mosmann and A. R. Williamson, data not shown). Further experiments involving various permutations of heavy and light chain mRNA fractions show that the amount of heavy chain rescued depends, in conditions of light chain excess (Fig. 4c), on the amount of heavy chain mRNA present in the oocyte. This argues against the stoichiometric assistance of some putative plasmacytoma helper protein in immunoglobulin secretion unless the respective mRNA exactly co-migrates with heavy or light chain mRNAs. Our data indicate that the X63 heavy chain is intrinsically non-secretory unless light chain is present. Such a situation occurs in variant lines which synthesize abnormal heavy chain and no light chain; the heavy chains are not secreted unless light chains are re-introduced through cell fusion methods⁹. What is the reason for this non-secretory phenotype? It might be argued that as a result of its known *in vitro* insolubility^{10–12}, intracellular heavy chain would aggregate in the absence of light chain and then be unavailable for secretion. The results shown in Fig. 2c show that a substantial proportion of the stranded heavy chain is in the form of covalent dimers and not the covalent aggregates which have been observed *in vitro*.

We conclude that the oocyte both assembles and secretes immunoglobulin; the assembly of IgG in oocytes has been noted by others¹⁸. If this immunoglobulin retains its antibody specificity, as seems likely¹⁹, the contribution of the individual chains to this specificity could be assessed by generating different combinations of heavy and light chain by means of mRNA injection. We do not understand why the separated heavy and light MOPC 21 chains are not secreted. The evidence presented above indicates that the chains exist in oocytes as monomers (light chain) or dimers (heavy chain) and we have preliminary evidence that they do not form strong attachments

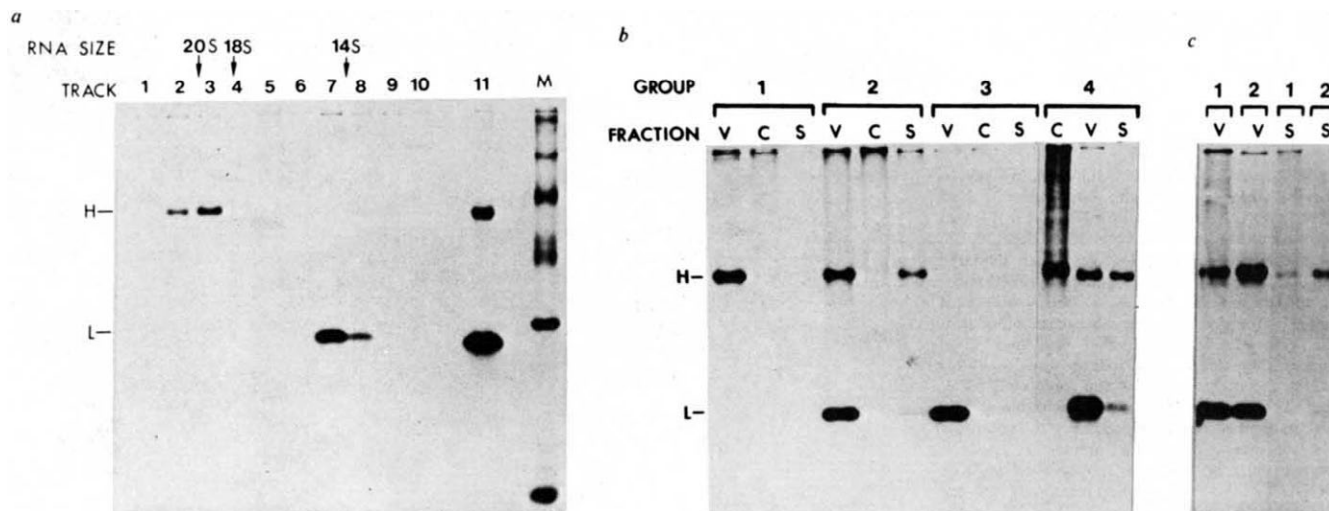


Fig. 4 Microinjection of separated heavy and light chain mRNAs. *a*, 30 μ g of poly(A) plus RNA (Fig. 1) were fractionated by electrophoresis (40 mA, 5 h, 4 °C) in 1.5% agarose gel (Sigma, type II, medium EEO) made in electrophoresis buffer (50% formamide, 0.1 mM EDTA, 3.6 mM Tris and 4 mM NaH_2PO_4 , pH 7.4); gel dimensions were $0.3 \times 16 \times 16$ cm. As MW marker, 5 μ g of *Xenopus laevis* total ovary RNA was also run on a neighbouring 8-mm wide slot. The part of the gel containing the X63 RNA was cut into 5-mm slices. Each slice (1 cm^2) was squeezed through a 23G needle into 1 ml of 10 mM Tris, 1 mM EDTA and 0.1% SDS, pH 7.5, and shaken at 4 °C for ~20 h. After removal of polymeric agarose by centrifugation at $180,000g_{\text{max}}$ for 30 min at 4 °C, poly(A)⁺ RNA from *X. laevis* ovary (a gift of P. C. Turner) was then added to each fraction as carrier. After ethanol precipitation the RNA pellet was resuspended in 30 μ l of distilled water and spun for 3 min in an Eppendorf microfuge before microinjection. Batches of oocytes were injected with carrier RNA (track 1), mRNA samples corresponding to consecutive fractions of the gel (tracks 2–10) or the original X63 mRNA (track 11) and then incubated for 24 h in ^{35}S -methionine. Alkylated immunoprecipitates prepared from oocyte homogenates were electrophoresed as described in Fig. 1 legend. The arrows at the top of the figures indicate the approximate size of the RNA injected as deduced from visualization with ethidium bromide of the track on the RNA gel containing the RNA marker. *b*, *c*, Oocytes were injected with fractionated or non-fractionated mRNAs as indicated below and cultured as described in Fig. 1 legend, then vesicle (V), cytosol (C) and incubation medium (S) fractions were immunoprecipitated, alkylated and electrophoresed on 12.5% polyacrylamide gels; *b* and *c* illustrate independent experiments. *b*, Group 1, heavy chain mRNA; 2, heavy chain plus light chain mRNAs; 3, light chain mRNA; 4, NS1 mRNA + heavy chain mRNA. *c*, Diluted (track 1) or undiluted (track 2) heavy chain mRNA + light chain mRNA. H, heavy chain; L, light chain.

to membranes. It is therefore possible that the non-appearance of plasmacytoma variants synthesizing only heavy chain is not caused by the aggregation of this protein. Thus, the rescue of heavy chain by light chain in oocytes might not be related to the reported mitigating effect of light chain on heavy chain aggregation *in vitro*¹².

As the H₂ dimer is known to be an intermediate form in the assembly of this subclass of immunoglobulins both *in vivo*²⁰ and *in vitro*¹², it will be interesting to see whether stranded H₂ dimers can be rescued by the subsequent injection of light chain mRNA. Such a demonstration would indicate that the topological block to secretion of isolated light chain occurs at the same point or further along the secretory pathway than the block to heavy chain secretion.

We thank Drs A. Williamson, C. Milstein and D. S. Secher for useful discussion, the Cancer Research Campaign for support, and S. Bhamra for technical assistance.

Received 4 December 1980; accepted 2 March 1981.

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Transcriptional regulation of mouse liver metallothionein-I gene by glucocorticoids

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Glucocorticoid hormones play an important part in the regulation of many essential metabolic processes¹. These include the synthesis of stress-related 'acute-phase' proteins² and the timing of developmental events in numerous tissues including fetal lung, liver and intestine, and adult mammary gland¹. Among the proteins that are induced by glucocorticoids during stress and fetal development are the metallothioneins (MT)^{3–6}. These small, cysteine-rich proteins are found primarily in the liver and kidney. They bind heavy metals and are thought to be involved in zinc homeostasis and heavy metal detoxification⁷. We show here that the accumulation of MT mRNA in mouse liver in response to the synthetic glucocorticoid, dexamethasone, is due to transcriptional activation of the metallothionein gene.

We began our study of MT expression *in vivo* by examining the kinetics of MT mRNA induction with dexamethasone. The probe used to quantitate mRNA levels was prepared by nick-translation of a cDNA clone containing the entire coding region of metallothionein-I (MT-I)⁸. The cDNA strand was separated from the message strand using a single-strand phage containing the MT-I message strand⁹. Initial experiments indicated that the kinetics of the MT-I mRNA induction were influenced by both the dose and the preparation of dexamethasone. A soluble form of dexamethasone elicited a rapid response, such that hepatic MT-I mRNA levels were maximal at 2 h and declined to basal levels by 6 h. With an insoluble, 'slow release' dexamethasone, MT-I mRNA levels rose gradually for 6–9 h. To obtain a MT-I

Table 1 Correlation between mRNA accumulation, transcription and the presence of specific glucocorticoid receptors

		Nuclear radioactivity (c.p.m. per μg DNA)		Nuclear receptors (molecules per cell)	MT-I mRNA (molecules per cell)	MT-I gene transcription (p.p.m.)
		50 μg Dex	5 mg Dex			
Liver	Control	—	—	—	13	3
	Dex	10.4 $\pm$ 1.49*	1.23 $\pm$ 0.24	59,500	1,360	260
Kidney	Control	—	—	—	14	4
	Dex	1.98 $\pm$ 0.56	0.61 $\pm$ 0.29	8,800	39	13

Six mice were each injected with ^3H -dexamethasone (Dex, 100 μCi ; 84 Ci mmol^{-1} ; NEN) and 50 μg of the soluble form of dexamethasone. Three of the mice also received a 100-fold excess (5 mg) of the soluble dexamethasone to monitor nonspecific nuclear binding of the ^3H -dexamethasone. After 2 h, liver and kidney nuclei were isolated and aliquots (400–1,000 μg of DNA) were counted at 35% efficiency. The DNA content of each sample was determined by a diphenylamine assay³². Receptor molecules per cell were calculated by subtracting column *b* from column *a* and using values of 770 mCi mmol^{-1} , 35% counting efficiency and 6.4 pg of DNA per cell. The mRNA levels were measured after 6 h of hormone treatment. The relative rate of transcription was determined after 2 h of hormone treatment (Fig. 3).

* Standard deviation ($n = 6$).

mRNA induction that was both rapid and prolonged, we used equal amounts of both seroid forms.

Figure 1 shows a composite dose response, based on several experiments using male and female mice of the Swiss Webster and BALB/c strains. Maximal induction of MT-I mRNA, measured at 6 h, was achieved with $>70 \mu\text{g}$ of dexamethasone.

The kinetics of MT-I mRNA induction with an optimal dose of dexamethasone (100 μg) are shown in Fig. 2. The MT-I mRNA level increased ~ 100 -fold to about 1,350 molecules per cell by 6 h and then gradually fell, presumably due to depletion of the steroid. We detected little difference in the kinetics or extent of mRNA accumulation between male and female mice in either mouse strain. Occasionally we observed a high basal level of MT-I mRNA in liver, sometimes as high as 300 molecules per cell, which is probably a stress phenomenon related to handling or cage conditions.

Steroids are generally assumed to act at the transcriptional level, although effects on mRNA stability have also been reported^{10,11}. Several different mRNAs are induced by glucocorti-

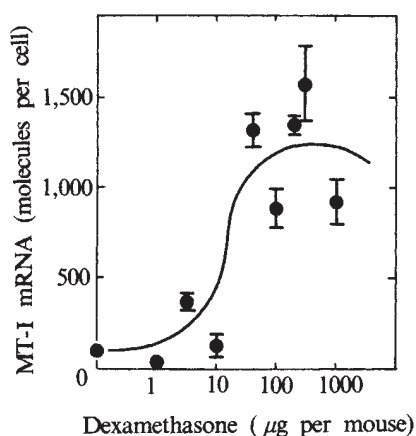


Fig. 1 Dose response of MT-I mRNA induction by dexamethasone. Mice (23–27 g) were injected subcutaneously with the indicated amount of dexamethasone or saline (0.2 ml). Fifty per cent of the dose was dexamethasone phosphate (Decadron; Merck, Sharp and Dohme) and 50% was dexamethasone acetate (Decadron-LA; Merck, Sharp and Dohme). At 6 h, the mice were killed and the livers were used to isolate total nucleic acid for mRNA measurements^{9,11}. MT-I mRNA levels were determined by hybridizing total nucleic acid samples to a ^{32}P -cDNA probe prepared by nick-translation of a cDNA clone which contains the entire coding region and 3'-untranslated region of MT-I⁸. The extent of hybridization was determined by S_1 nuclease resistance and compared with a standard curve generated from the hybridization of the probe to known amounts of a recombinant bacteriophage fd DNA containing the message strand of MT-I⁹. Results are reported as mRNA molecules per cell, assuming a molecular weight of 130,000 for MT-I mRNA and that mouse liver cells contain 6.4 pg of DNA. Error bars show the standard error ($n = 3$ –9) for each point.

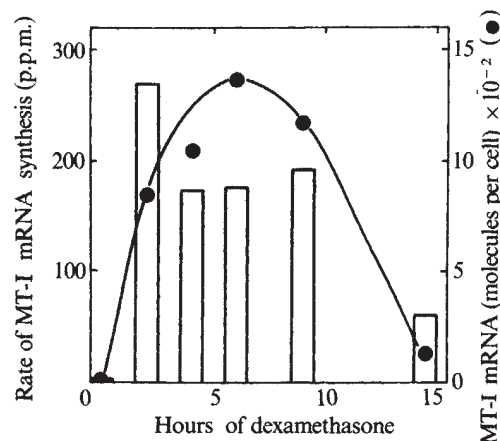


Fig. 2 Time course of MT-I mRNA accumulation and the relative rate of transcription in mouse liver. Tissue from mice induced for various times with 100 μg of dexamethasone was used to measure mRNA levels as in Fig. 1 (●). Nuclei from the same tissue samples were isolated and transcribed *in vitro* in the presence of [α - ^{32}P]UTP¹¹. ^{32}P -RNA transcripts were isolated and three different amounts of each sample were hybridized to plasmid DNA immobilized on nitrocellulose filters¹¹. Each hybridization reaction contained a filter with 0.7 μg of $m_1\text{pSH}_{1.3}$ (a plasmid containing a 1.3-kilobase genomic DNA insert representing the entire MT-I gene^{8,9}) and a control filter with 0.7 μg of pBR322. Relative rates of transcription (histograms) are reported as parts per million (p.p.m.) and were derived from the slope of the line generated by plotting radioactivity hybridized versus total input radioactivity (see Fig. 3). Background (<10 p.p.m.) measured by the pBR322 filters, was subtracted. Values are corrected for 44% hybridization efficiency, as determined by the hybridization of MT-I mRNA in a parallel reaction.

coids, including those coding for tyrosine aminotransferase¹², tryptophan oxygenase¹³, ovalbumin¹⁴, transferrin¹⁵, growth hormone¹⁶, $\alpha_2\mu$ -microglobulin¹⁷ and metallothionein^{18,19}. However, the only direct demonstrations of transcriptional control by glucocorticoids are for mammary tumour virus expression^{20,21} and possibly casein gene expression²². To ascertain whether MT-I mRNA accumulation is a consequence of transcriptional activation, liver nuclei were isolated at various times after hormone administration and the endogenous RNA polymerases were allowed to transcribe a few hundred nucleotides in the presence of [α - ^{32}P]UTP. The ^{32}P -RNA was hybridized to immobilized plasmid DNA containing the genomic MT-I sequence. This assay essentially measures the relative number of RNA polymerases on the MT-I gene at the time the nuclei are isolated¹¹. The histograms in Fig. 2 show that the relative rate of transcription of the MT-I gene increased ~ 90 -fold within the first 2 h and stayed high for another 7 h. Similar effects of dexamethasone on MT mRNA synthesis are observed with cultured mouse cells²³. The kinetics of transcriptional activation predict the mRNA levels reasonably well,

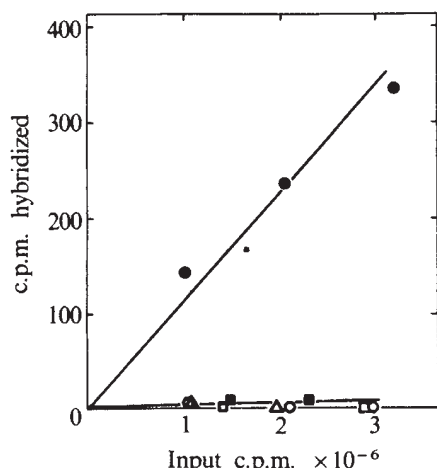


Fig. 3 Effect of α -amanitin on MT-I gene transcription. Liver nuclei from mice induced with dexamethasone for 2 h were transcribed in the presence of [α - 32 P]UTP (●); duplicates included $5 \mu\text{g ml}^{-1}$ α -amanitin (△). Varying amounts of 32 P-RNA transcripts were hybridized as described in Fig. 2 legend. pBR322 controls have been subtracted. Also shown are the results of the transcription of uninduced liver nuclei (○), and kidney nuclei from induced (■) and uninduced (□) mice.

although small effects on mRNA stability cannot be entirely ruled out.

Figure 3 illustrates the effect of α -amanitin on the relative rate of MT-I gene transcription. When liver nuclei isolated 2 h after dexamethasone treatment were allowed to transcribe in the presence of α -amanitin, total transcription was inhibited by 54% and specific MT-I transcription dropped from 260 p.p.m. to less than 10 p.p.m. Thus, we conclude that MT-I mRNA is transcribed by RNA polymerase II (form B), as are all other mRNAs.

Heavy metals such as zinc and cadmium also induce mouse liver MT-I mRNA by transcriptional activation²⁴. Maximal levels of transcription and mRNA accumulation (2,200 molecules per cell) are 1.5- to 2-fold higher than those obtained with dexamethasone; thus, there is a good correlation between the rate of transcription and mRNA accumulation with both metal and hormonal inducers. The ratio of the amount of MT-I protein that accumulates in the liver to the amount of MT-I mRNA induced by glucocorticoids or metals has not been determined adequately, primarily because of the difficulty in achieving steady-state conditions *in vivo*.

With dexamethasone, MT-I mRNA induction is most prominent in liver but we were also able to measure low levels in other tissues such as heart, skeletal muscle, spleen and kidney. MT-I mRNA accumulation and transcription are barely detectable in the kidney (Fig. 3, Table 1), whereas this tissue accumulates about 700 molecules of MT-I mRNA per cell in response to cadmium²⁴. To explore the basis of this difference in responsiveness, we measured the number of nuclear glucocorticoid receptors in the liver and kidney. Table 1 shows that there are 6.5-fold more nuclear glucocorticoid receptors (59,500 molecules per cell) in the liver than in the kidney (8,800 molecules per cell). The number of nuclear glucocorticoid receptors found in the liver is consistent with the number of total cytoplasmic receptors ($\sim 10^5$ per cell) calculated from the data of Wrangé *et al.*²⁵. Assuming that mRNA induction is proportional to nuclear receptor levels²³, the paucity of glucocorticoid receptors accounts largely for the meagre response of the kidney relative to the liver. In addition, the data suggest that a lower percentage of the cells in the kidney than the liver may be responsive to either inducer. The minimal response of the kidney to glucocorticoids is consistent with our observations, as well as those of Oh *et al.*³, that stressed animals have high levels of MT-I mRNA and protein in the liver but not in the kidney.

The finding that MT genes are under glucocorticoid control may be explained in several ways. The available data suggest that the apoproteins synthesized from MT mRNA bind zinc predominantly; thus, induction of these proteins may increase the intracellular pool of this essential metal^{13,19,26}. Recent studies indicate that MT may transfer zinc directly to other zinc-requiring apoenzymes^{27,28}. A requirement of zinc for rapid tissue growth may account for the high levels of fetal MT that occur just before parturition^{4,5}. Notably, the induction of fetal MT coincides with a dramatic rise in circulating glucocorticoids^{29,30}. Because many heavy metals will displace zinc from MT³¹, the induction of zinc-MT by glucocorticoids could also provide insurance against subsequent exposure to other toxic metals.

This work was supported by NIH grant HD-09172.

Received 15 December 1980; accepted 12 March 1981.

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Unilateral internuclear gene transfer and cell differentiation in *Schizophyllum*

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The recent proliferation of studies on moveable genetic elements has suggested that genome rearrangement can contribute to the underlying mechanisms involved in cell differentiation¹. Evidence to support this notion is rapidly accumulating in both prokaryotic and eukaryotic organisms²⁻⁵. So far, however, relatively few eukaryotes have lent themselves to genetic analysis with resolution sufficient for the detection of genome rearrangement during cell differentiation. The filamentous fungi as exemplified here by *Schizophyllum commune*, provide an unusual experimental opportunity for detecting moveable genetic elements in that the organism grows as a binucleate heterokaryon. Each cell contains two compatible haploid nuclei

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which can be experimentally dissociated into uninucleate cells, and these cells can then be used in genetic analysis to detect genetic changes. We now present evidence that nuclei in cells from differentiated mound bodies show a specific genetic change whereas nuclei from surrounding non-mound vegetative cells show no detectable genetic changes. The binucleate cells of differentiated mound bodies were found to contain haploid nuclei one of which remained unchanged with regard to its genetic marker genes whereas the other nucleus, and always the same one, contained specific marker genes from the other nucleus. A unilateral transfer of gene copies between nuclei thus seems to be associated with mound cell differentiation. The genetic change is not the result of mutation and also is distinguished from somatic recombination⁶, but is similar to single factor transfer⁷. The transferred copies are stably integrated into the recipient genome and subsequently behave as mendelian genes.

S. commune grows as a dikaryon, cells with two compatible haploid nuclei, and genetic techniques have been well developed such that the two nuclei can be marked with a variety of gene mutations of known linkage relationships⁸. Eventually, cell differentiation occurs in various areas of the dikaryotic colony, resulting in the formation of multicellular, aerial reproductive structures. The two haploid nuclei can be induced to dissociate and form uninucleate cells when grown on a bile salt-containing medium⁹. Individual nuclei of dikaryons recovered in this way can be crossed with tester strains and their constellation of genetic markers determined. Genetic analysis involves collecting meiotically produced spores from the reproductive structures and carrying out random spore genetic analysis.

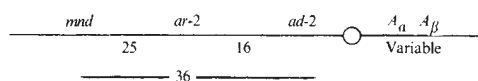


Fig. 1 *mnd* linkage group with loci relevant to the present study.

The evidence reported here involves the recessive mutant allele *mnd*, whose phenotype is observed as abnormal growths called 'mounds'¹⁰. These structures develop in some monokaryotic strains which possess the *mnd* allele and in all dikaryotic strains that are homoallelic for *mnd*⁹. Mounds are characteristically large aerial hemispherical bodies of compacted hyphae that exhibit properties of autonomous and indeterminate growth. At present it is not clear whether mounds represent abnormal reproductive structures (the mushroom-like fruiting bodies) or abnormal vegetative growth. We have previously presented evidence that mounds which develop on an initially heteroallelic (*mnd* + *mnd*⁺), dikaryotic colony are composed of homoallelic (*mnd* + *mnd*) dikaryotic cells and that non-mound mycelium remaining in such colonies is composed of heteroallelic cells⁹.

The behaviour of the *mnd* gene in dikaryons heteroallelic for *mnd* was examined in relation to other genes both linked and unlinked to *mnd*. The linkage relationship of the mutant alleles, *ad-2* and *ar-2*, to the *A* mating genes have been previously established¹¹. The linkage relationships of *mnd* to *ad-2* and *ar-2* shown in Fig. 1 were generated by analysis of 130 random spore progeny from the cross: *A41 B41 mnd*⁺ *ad-2 ar-2* × *A47 B42 mnd ad-2*⁺ *ar-2*⁺ (data not shown). Other mutant alleles used, *ni-3*, *ar-6* and *ur-1* as well as the *B* mating type locus, are located on separate chromosome arms¹¹ and are consequently unlinked to *mnd* and to each other (Table 1). The methods for scoring *mnd*, mating type genes and auxotrophic marker genes have been described previously⁹.

A replicate series of 55 crosses of the type P1 (*A47 B42 mnd ad-2 ni-3 ur-1*⁺ *ar-6*⁺) × P2 (*A43 B41 mnd*⁺ *ad-2*⁺ *ni-3*⁺ *ur-1 ar-6*) produced the expected dikaryotic colonies on which mounds developed. Mound tissue was subcultured from each of these colonies and grown on a medium containing bile salts to form numerous nuclear dissociations producing uninucleate cells. All the recovered monokaryons fell into two types (Table 1). One type was genetically identical to the original *mnd* parent

Table 1 Monokaryotic component strains recovered from mound and non-mound dikaryotic cells

Dikaryotic nuclei: P1 (*A47 B42 mnd ad-2 ni-3 ur-1*⁺ *ar-6*⁺) and P2 (*A43 B41 mnd*⁺ *ad-2*⁺ *ni-3*⁺ *ur-1 ar-6*)
Recovered monokaryotic cells*

From mound tissue	No. recovered	From non-mound tissue	No. recovered
Nuclear types:		Nuclear types:	
<i>A47 B42 mnd ad-2 ni-3 ur-1</i> ⁺ <i>ar-6</i> ⁺	418	<i>A47 B42 mnd ad-2 ni-3 ur-1</i> ⁺ <i>ar-6</i> ⁺	361
<i>A43 B41 mnd</i> ⁺ <i>ad-2</i> ⁺ <i>ni-3</i> ⁺ <i>ur-1 ar-6</i>	132	<i>A43 B41 mnd</i> ⁺ <i>ad-2</i> ⁺ <i>ni-3</i> ⁺ <i>ur-1 ar-6</i>	129

Genetic marker abbreviations used above and in Fig. 1. *mnd*, Mound allele; *ni-3*, nicotinamide requiring; *ar-6*, arginine requiring; *ad-2*, adenine requiring; *ur-1*, uracil requiring. *A* factor, the *A* incompatibility factor which regulates aspects of the dikaryotic phenotype; *B* factor, the *B* incompatibility factor which regulates aspects of the dikaryotic phenotype.

* Recovered strains designated *mnd*⁺ were originally the *mnd*⁺ component of the parent dikaryon, but are now *mnd*. Similarly for *ad-2*⁺.

strain (P1). The other was genetically similar to the *mnd*⁺ parent strain (P2) with the exception that it had acquired *mnd* and *ad-2* alleles apparently replacing the corresponding alleles originally present. It thus seems that there is a donor nucleus (*mnd*) and a recipient nucleus (initially *mnd*⁺) with respect to the genetic transfer that occurred. In addition, only genes linked to *mnd* showed translocation.

The frequency with which genes linked to *mnd* co-transferred with *mnd* in dikaryotic mound tissue was examined in subcultures from 125 replicate crosses of the type *A51 B51 mnd ad-2 ar-2* × *A41 B41 mnd*⁺ *ad-2*⁺ *ar-2*⁺. Each of the 125 mound isolates was scored for homozygosity of genetic markers as observed by growth on appropriately substituted minimal media. As expected, each of the mound isolates had become homozygous for the *mnd* allele and 115 of the isolates had also become homozygous for the *ad-2* and *ar-2* alleles. The other 10 mound isolates remained heterozygous for these alleles. The mating type loci also remained heterozygous in all the isolates, as indicated by the continued dikaryotic state of the nuclei within the cells. Thus, the co-transfer of genes linked to *mnd* is frequent but variable: in 9% of the above events the markers linked to *mnd* were not observed to co-transfer with *mnd*.

We have also observed in other crosses that 'co-transferred' genes can be either wild type or mutant, that is, *ar-2*⁺ and *ad-2*⁺ have also replaced their allelic counterparts when co-transferred with *mnd* (unpublished). Such evidence makes it unlikely that it is a *trans*-acting 'signal' that is transferred, that is, a signal from *mnd* which converts *mnd*⁺ to *mnd*, by something akin to chromosomal 'inactivation'.

Although a positive selection for the transfer of genes in phenotypically heteroallelic non-mound vegetative cells has not been developed, the sampling and testing of thousands of these cells have not revealed the transfer of *mnd* or any other marked genes^{9,12}. The development of a selection system using recessive drug-resistance markers is in progress to discern whether there is any transfer of genes from *mnd*⁺ nuclei or if this is strictly a *mnd*-dependent unidirectional transfer.

We conclude that in the binucleate heterokaryon phase of *S. commune* there is a process, as yet uncharacterized, by which copies of genes from one nucleus can be unilaterally transferred to the other nucleus of the dikaryon. The transferred genes seem to substitute for their corresponding alleles in the recipient nucleus, and subsequently have been demonstrated to behave in a mendelian fashion^{9,12}. The previous alleles in the recipient nucleus are apparently lost. The donor nucleus is not detectably altered during the transfer process. All these observations suggest that gene transfer involving the *mnd* region of the *A* mating factor chromosome occurs without nuclear fusion.

This work was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and by NSF grant PCM77-05581 to T.J.L.

Received 2 January; accepted 16 March 1981.

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A small RNA complementary to an intervening sequence is produced late in SV40 infection

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The post-transcriptional modifications that affect simian virus 40 (SV40) mRNA are similar to those undergone by most eukaryotic mRNAs and include 5'-end capping, 3' polyadenylation and splicing of mRNA precursors¹. Late in SV40 infection unspliced 19S RNAs are found predominantly in the nucleus whereas the spliced 19S RNAs are mainly cytoplasmic^{2,3}. Nuclear and cytoplasmic 19S RNAs differ in that all the cytoplasmic species have lost a 32-nucleotide intervening sequence located between 0.760 and 0.765 map units^{3,4}. Previous transcriptional studies of SV40 deletion mutants implied that splicing of the late nuclear transcripts and transport of RNA into the cytoplasm were coupled events⁵. More recent studies, however, demonstrate that transport of unspliced 19S RNA can occur when the sequences encompassing the 32-nucleotide intervening sequence (0.760–0.765 map units) are deleted⁶. We have previously proposed that this small 19S RNA intervening sequence may be involved in the retention of the unspliced RNA within the nucleus³. One hypothesis to account for recognition of the 32-nucleotide intervening sequence by a nuclear retention system postulates the existence of a small nuclear RNA complementary to the intervening sequence. We describe here the initial characterization of a small nuclear RNA made late in SV40 infection which is complementary to the DNA of the 19S intervening sequence. Several host cytoplasmic RNAs also hybridize to this intervening sequence.

The initial experimental step was the construction of a hybrid plasmid DNA carrying the 32-nucleotide intervening sequence (IVS) DNA from SV40 DNA (0.760–0.765 map units), and removal of the 32-base pair fragment after appropriate restriction enzyme cleavage to the pBR325–DNA–plasmid vector (Fig. 1). The resulting product was called PBR325/IVS and was attached to diazobenzoyloxymethyl (DBM)-paper according to the procedure described by Alwine *et al.*⁷. RNA present in the nucleus of SV40-infected cells during the late infection phase was extracted by an isotonic method^{3,8}. This nuclear RNA was labelled at the 5' end⁹ and hybridized against the PBR325/IVS DNA attached to the DBM-paper. Hybridized RNA was eluted and analysed by electrophoresis in a 12% acrylamide gel in denaturing conditions (8 M urea). The results (Fig. 2) show the presence of a small RNA of ~60 nucleotides which is present in the nucleus of infected cells but not in that of uninfected cells (Fig. 2, lane a). In a control experiment using vector DNA

without IVS DNA attached to DBM-paper, no RNA was retained by the paper (data not shown). The sensitivity of this small RNA to digestion with RNase is shown in Fig. 3, lanes a, b. These results indicate that the small RNA found in the nucleus of SV40-infected cells is in fact complementary to the IVS DNA sequence. In addition to the 60-nucleotide RNA (henceforth referred to as *ivsRNA*), the nuclear fraction contains a very minor RNA band corresponding to ~38 nucleotides.

The existence of small RNA molecules within the nucleus (snRNA) of eukaryotic cells is well established and many of these snRNAs have been characterized^{10,11}. However, the induction of a small nuclear RNA complementary to an intervening sequence has not been previously described. The function of the snRNAs is not established, although it has been proposed that they are involved in the mRNA splicing process.

The small virus-induced nuclear RNA described here possesses two marked differences from previously described snRNAs: it is generally smaller, 60 compared with ~200 nucleotides, and it does not have a 5'-terminal cap, as this *ivsRNA* can be labelled at the 5' end. Although post-transcriptional cleavage should be considered, this suggests that the synthesis of the SV40 sRNA differs from that of most eukaryotic snRNAs.

Our hybridization selection procedure for the small RNAs did not use sequences adjacent to the 32-nucleotide intervening sequence, and the conditions of hybridization are reasonably stringent. Therefore, due to the short length of the hybrids, all the small RNAs we described must have almost complete homology to the intervening sequence. Although our search for these small RNAs stems from the prediction of a recognition system for the retention of nuclear transcripts, their existence does not necessarily confirm this hypothesis and other explanations are possible^{12–14}.

The subcellular distribution of small RNAs complementary to the intervening sequence is also shown in Fig. 2. The 60-nucleotide sRNA is clearly more abundant in the nuclear fraction. The paranuclear fraction also contains the 60-nucleotide

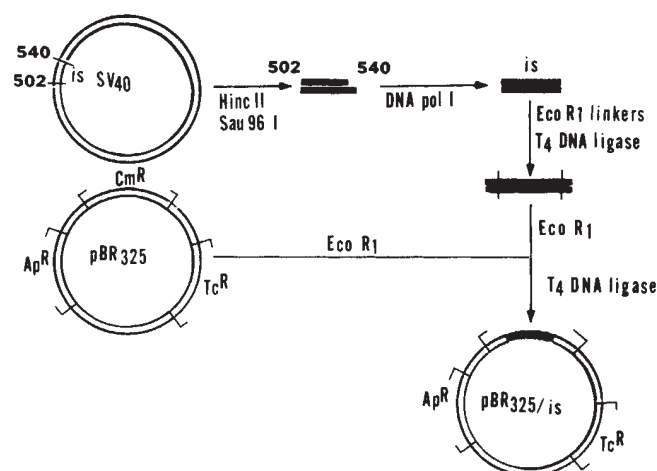


Fig. 1 Scheme for the construction of pBR325/IVS: SV40 DNA was cut with *HincII* and *Sau96I* restriction enzymes, and the small 38-base pair fragment generated by this cleavage was gel purified. After electroelution from the gel, the fragment was treated with DNA polymerase I to fill the *Sau96I* terminus as described elsewhere¹⁸. *EcoRI* linkers were joined to the 38-base pair fragment with *T4* ligase, generating a 50-base pair fragment¹⁹, and the pBR325–DNA and the 50-base pair fragment DNA cut with *EcoRI*. The 5'-cohesive termini were joined to each other with *T4* ligase as previously described²⁰. The plasmid DNAs were used to transform *Escherichia coli* HB101 cells²¹, and the transformants selected for growth in the presence of ampicillin. Transformed cells carrying pBR325/IVS DNA were then selected for loss of chloramphenicol resistance. The structure of the plasmid DNA in candidate colonies was confirmed by further restriction analysis and subsequently by DNA sequencing according to Maxam and Gilbert⁹.

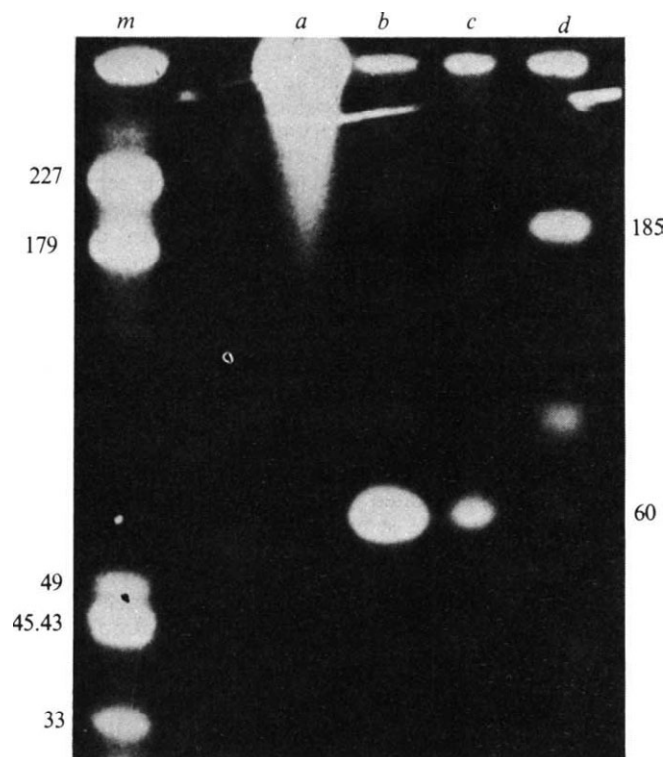


Fig. 2 Gel analysis of ivsRNA. Semiconfluent monolayers were infected at a high multiplicity of infection (MOI = 10), and 48 h later RNA was extracted using isotonic procedure as described previously^{3,8}. Briefly, the cell monolayers, after washing with TSM (30 mM Tris-HCl pH 7.6, 150 mM NaCl, 1.5 mM MgCl₂) and lysis with TSM plus 0.2% NP40 on ice, were scraped off the plates and centrifuged (1,000g, 3 min). The cytoplasmic supernatant was decanted and the residual nuclei removed by a second centrifugation. The 1,000g pellets (nuclear and paranuclear fraction) were resuspended with TSM-NP40, pelleted again and resuspended with TSM-NP40, 0.2%, plus 0.05% deoxycholate and centrifuged again at 1,000g. This supernatant (paranuclear fraction) was decanted and the nuclear pellet resuspended in TSM-NP40. All fractions were mixed with extraction buffer (10 mM Tris-HCl pH 7.8, 1 mM EDTA, 350 mM NaCl, 2% SDS and 7 M urea), extracted twice with H₂O and saturated 1:1 phenol/chloroform and ethanol precipitated. Extracted RNAs were digested with DNase³, dephosphorylated, 5'-end labelled using [γ -³²P]ATP⁹ then ethanol precipitated. Dry pellets from each fraction were resuspended in hybridization buffer (50% formamide, 100 mM PIPES, pH 6.8, 600 mM NaCl, 1 mM EDTA, 0.1% SDS) and hybridized against pBR325/IVS DNA fixed to DBM-paper preformed as described elsewhere⁷. After 48 h at 45 °C the hybrids were washed at 45 °C three times with 40% formamide, 4 × SSC, 0.1% SDS and three times more with 2 × SSC at room temperature. RNA elution from the pBR325/IVS DNA-paper was done twice at 65 °C for 15 min with 100% formamide and the resultant eluted material was precipitated with ethanol. The pellets were resuspended in TE (10 mM Tris-HCl pH 7.6, 1 mM EDTA) and equal volumes of formamide-dye mixture were added. After 2–3 min at 100 °C, the samples were loaded on a 12% acrylamide gel with 8 M urea in TBE (50 mM Tris-borate pH 8.3, 1 mM EDTA) and run for 14–16 h at 85 V. Wet gels were exposed with Kodak XR-5 film at –70 °C for 24 h. *a*, Nuclear RNA from uninfected cells; *b*, nuclear RNA from infected cells; *c*, paranuclear RNA from infected cells; *d*, cytoplasmic RNA from infected cells; *m*, SV40 *Hae*III markers.

species but at substantially reduced levels. The small RNA species present in the cytoplasmic fraction differ significantly from those in the nucleus; the most abundant is 185 nucleotides long and is present in uninfected cells. Several minor species (see Fig. 3) of ~160, 87, 77 and 73 nucleotides length are also observed. The major cytoplasmic species is much longer and

more abundant than the nuclear species (185 compared with 60 nucleotides). Because the cytoplasmic ivsRNAs are all longer than the nuclear species, and are mostly present in uninfected cells, leakage of nuclear species cannot account for any of the cytoplasmic ivsRNAs seen. The function of these RNAs is unknown but their presence in the cytoplasm may imply an involvement with the translational machinery. Small cytoplasmic RNAs have previously been observed during adenovirus¹⁵ and SV40 infection¹⁶. A 65-nucleotide cytoplasmic RNA that arises late in SV40 infection and is complementary to early mRNA at 0.21 map units (SAS RNA) has been described by Alwine and Khoury¹⁷, but unlike the nuclear ivsRNA described here, their SAS RNA has no apparent association with splice junctions or intervening sequences. Furthermore, the existence of a small RNA which will hybridize to SV40 DNA near or at 0.76 map units has been independently observed by others (J. Alwine, personal communication). Clearly, more information on the sequence and function of these SV40 ivsRNAs is necessary to establish their role during infection.

We thank Susan Carr for technical assistance. R. C. is a fellow from the Consejo de Investigaciones Científicas Y Técnicas, Republica Argentina. This work was supported by PHS grant CA-25819 from the NCI to L.P.V.

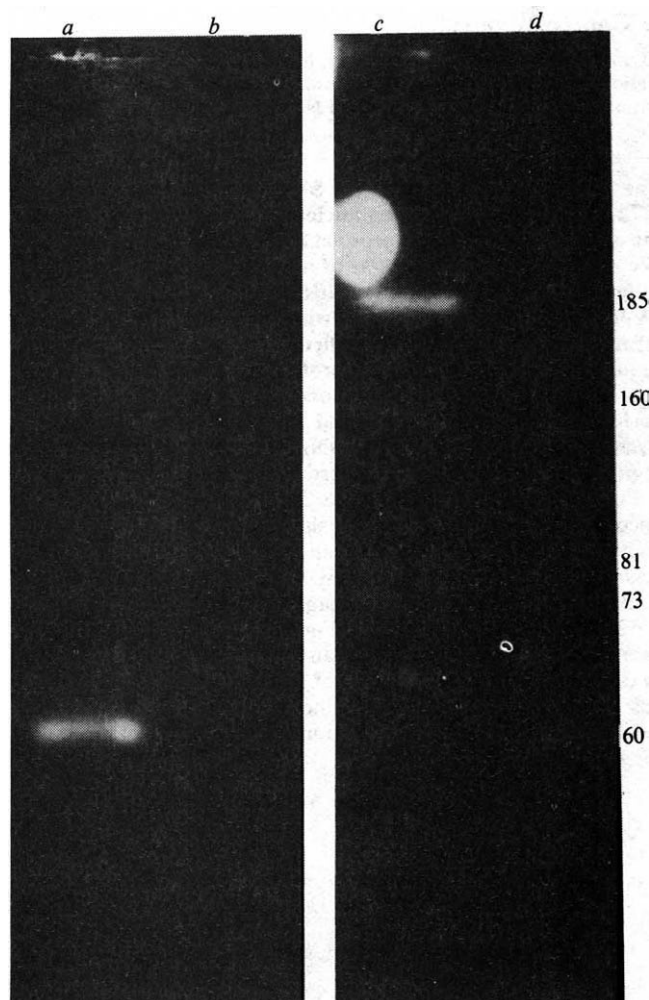


Fig. 3 RNase sensitivity of small RNAs. RNAs from nuclear and cytoplasmic fractions of infected cells were extracted and selected as described in Fig. 2 legend. Nuclear and cytoplasmic small RNAs were then treated with RNase (RNase A, 0.1 mg ml⁻¹, RNase T, 50 U ml⁻¹, 37 °C, 45 min) in 10 mM MgCl₂, 10 mM Tris-HCl pH 7.6, 2.5 mM dithiothreitol and 0.25 mg ml⁻¹ gelatin. *a*, Nuclear RNA; *b*, nuclear RNA plus RNase; *c*, cytoplasmic RNA; *d*, cytoplasmic RNA plus RNase.

Received 3 February; accepted 11 March 1981.

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Identification of the SV40 agnogene product: a DNA binding protein

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The proximal late region of the SV40 genome (map positions 0.72-0.76) contains an open nucleotide reading frame^{1,2} called the agnogene³ because its product had so far not been detected. We report here the detection of a novel protein of molecular weight (M_r) 7,900 (61 amino acids) in cells lytically infected by SV40; its identity has been confirmed by comparing the amino-terminal sequence with that predicted from the SV40 nucleotide sequence. This so-called agnoprotein accumulates late in the lytic cycle and has a relatively short half life of ~2 h. The highly basic nature of the protein and its affinity for both single-stranded and double-stranded DNA suggests that it may have a regulatory role in nucleic acid-protein interactions.

For the SV40 genome, up to 20% of the DNA sequence encodes no known protein^{1,2}. A significant portion of this non-coding sequence seems to exist in regulatory structures but at least one segment in the proximal late region of the genome contains an open reading frame (agnogene) capable of encoding a 62-amino acid polypeptide. Figure 1 shows the agnogene, located between positions 0.72 and 0.76 on the SV40 genome (a) and the amino acid sequence of its product (b). The sequence has a large number of the amino acids arginine, lysine and leucine; the only methionine codon present is that of the initiator AUG.

To detect this protein product, SV40-infected African green monkey kidney cell cultures were labelled with ¹⁴C-arginine, and proteins were extracted late in the lytic cycle. Figure 2 shows a protein band migrating in the position expected for the agnoprotein (7,900 M_r) in wild-type SV40-infected cells, which is absent from both mock-infected cells and cells infected by a viable deletion mutant of SV40 (dl 2304) which contains a deletion over the entire region of the agnogene (unpublished data). A protein migrating in the same position was detected when cells were labelled with ¹⁴C-leucine, but not with ³⁵S-methionine (data not shown).

Two types of experiment suggested that the 7,900 M_r (7.9K) polypeptide observed in Fig. 2 was in fact the agnoprotein: (1) cell-free translation of late lytic SV40 RNA selected by hybridization to SV40 DNA-containing filters gave a polypeptide which co-migrated with the species identified in Fig. 2 (data not shown). (2) A two-nucleotide insertion mutant of SV40 which shifts the reading frame for the putative agnoprotein produced an appropriately truncated polypeptide (data not shown). Amino-terminal peptide sequencing (Fig. 3) provided direct evidence for the identity of the agnogene product. Extracts from lytically infected monkey kidney cells, labelled with either ¹⁴C-leucine or ¹⁴C-arginine, were prepared by affinity chromatography, followed by SDS-polyacrylamide gel electrophoresis; the 7.9K band was eluted and subjected to N-terminal sequence analysis⁴. An amino acid sequence through the first 16 Edman cycles confirms the identity of the agnogene product. The observed sequence, xxx-Leu-Arg-Arg-Leu-xxx-Arg, is present only once in the entire viral genome, at the beginning of the agnogene. An almost quantitative recovery of radiolabelled counts suggests that little if any of the polypeptide contains a blocked amino terminus. Furthermore, the positions of the confirmed amino acids compared with the putative sequence of the polypeptide (Fig. 1) indicate that the initiating methionine is quantitatively removed, leaving valine as the N-terminal amino acid.

To investigate the time course of appearance of the 7.9K polypeptide, monkey kidney cell cultures were labelled at various times after infection (see Fig. 4); the polypeptide appeared only late after lytic infection and thus seems to parallel the appearance of the virion polypeptides (Fig. 4a). For comparison, the SV40 tumour antigens were immunoprecipitated from the same set of samples using a broad spectrum anti-tumour serum (Fig. 4b). Although the large T and small t antigens seem to be synthesized in greater amounts late in infection, their presence is also easily detected at early stages. As it was conceivable that some agnoprotein was made early in the lytic cycle at levels below our detection, we tried to find an antiserum to the agnoprotein to increase the efficiency of its detection. None of the available antisera, including different anti-tumour sera and sera produced against SDS-disrupted virions, were able to immunoprecipitate the agnoprotein.

Data from pulse-chase experiments performed 48 h post-infection (not shown) indicate that the 7.9K polypeptide decays

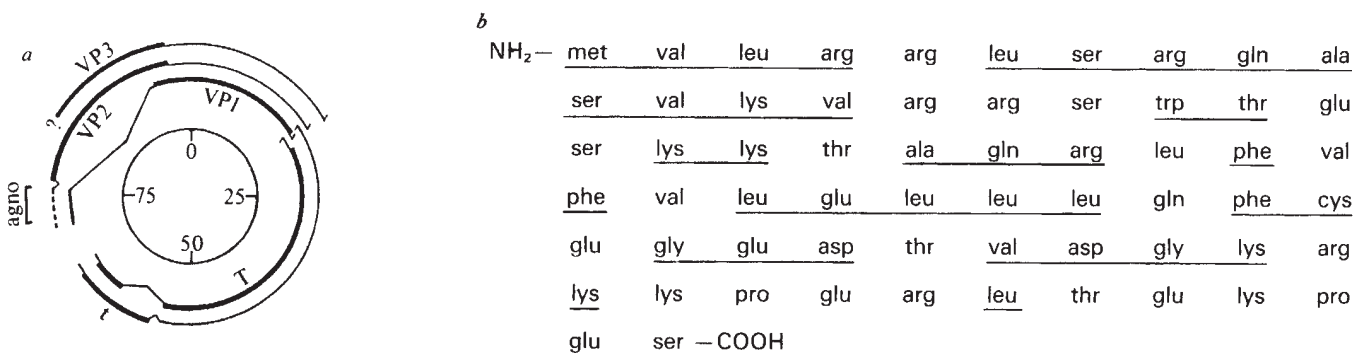


Fig. 1 Coding region and amino acid sequence of the putative product of the SV40 agnogene. *a*, Map location of the agnogene relative to the known SV40 mRNAs. The heavy lines indicate the coding sequences within each of the mRNAs. *b*, Amino acid sequence of the agnogene product as deduced from the known nucleotide sequence. Amino acid residues which are shared with the BKV agnogene product are underlined.

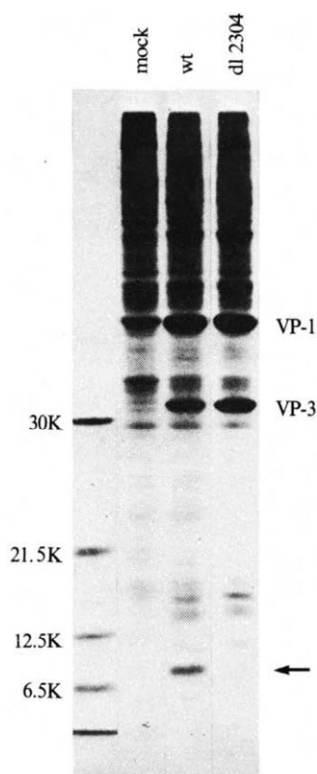


Fig. 2 Identification of the SV40 agnogene product. Secondary cultures of African green monkey kidney (AGMK) cells were infected with either wild-type (wt) SV40 or a deletion mutant dl 2304, at a multiplicity of 10–20 plaque-forming units (PFU) per cell. At 48 h post-infection, the cultures were radiolabelled with ^{14}C -arginine ($350 \text{ mCi mmol}^{-1}$) at $16.7 \mu\text{Ci ml}^{-1}$ for 2 h. Cells were collected and lysed in buffer A (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, $25 \mu\text{g ml}^{-1}$, L-1-tosylamide-2-phenylethylchloromethyl ketone). After centrifugation at $100,000 \text{ g}$ for 30 min, the supernatant was analysed on a 12.5% SDS-polyacrylamide gel as described elsewhere²⁰. The gel was fluorographed and dried, and an autoradiogram obtained by exposing the dried gel to Kodak XR-2 films at -80°C . The molecular weight markers used were carbonic anhydrase (30 K), soybean trypsin inhibitor (21.5 K), cytochrome *c* (12.5 K) and aprotinin (6.5 K). The arrow indicates the position of the agnoproduct.

with a half life of $\sim 2 \text{ h}$ which contrasts with that of T antigen ($\sim 10 \text{ h}$) and VP-1 (15 h) determined in parallel. This short half life, together with the relative ease of labelling of the agnoproduct, suggest a rapid synthetic rate and indicate a potential regulatory function rather than a structural role for the agnoproduct.

The highly basic nature of the agnoproduct (arginine + lysine = 25%) suggested that it may bind to DNA and/or RNA; thus a binding assay was done using single-stranded (ss) and double-stranded (ds) calf thymus DNA with lytically infected cell extracts which contained both the agnoproduct and the known DNA binding protein, SV40 T antigen^{5–8}. Figure 5a shows that almost all the input agnoproduct was retained on either ss or dsDNA columns at low salt concentration (0.1 M NaCl) and could be quantitatively eluted with high salt (1.0 M NaCl). In contrast (Fig. 5b) $\sim 50\%$ of the SV40 T antigen did not bind to either the ss or dsDNA in identical conditions (0.1 M NaCl). None of the SV40 T antigen (previously shown not to be a DNA binding protein⁹) was retained in these conditions. Thus, unlike SV40 T antigen, which seems to be composed of heterogeneous populations with respect to DNA binding properties, the agnogene protein homogeneously and quantitatively binds both ss and dsDNA. The agnoproduct, and the bound fraction of T antigen, elute at 0.3 M NaCl (data not shown).

These data indicate that the SV40 agnoproduct is synthesized in large quantities late in the lytic cycle and that has a remarkably short half life. The observation that mutants with deletions within the agnogene region are viable^{10–14} suggests that, at least in a tissue culture system, the agnoproduct, like the T antigen encoded by the early region of SV40, is not essential. However, several studies have shown that mutants with deletions in this region often do not grow as well as wild-type virus¹⁵. A small genome like SV40, subject to rapid evolutionary pressures, would not contain sequences which were of little or no advantage. Thus we can speculate that the agnoproduct may act simply to enhance the efficiency of SV40-specific functions. Alternatively, its role in tissue culture systems may be substituted by a host cell protein (albeit with a lower efficiency). A significant role for the SV40 agnoproduct is suggested by the conservation

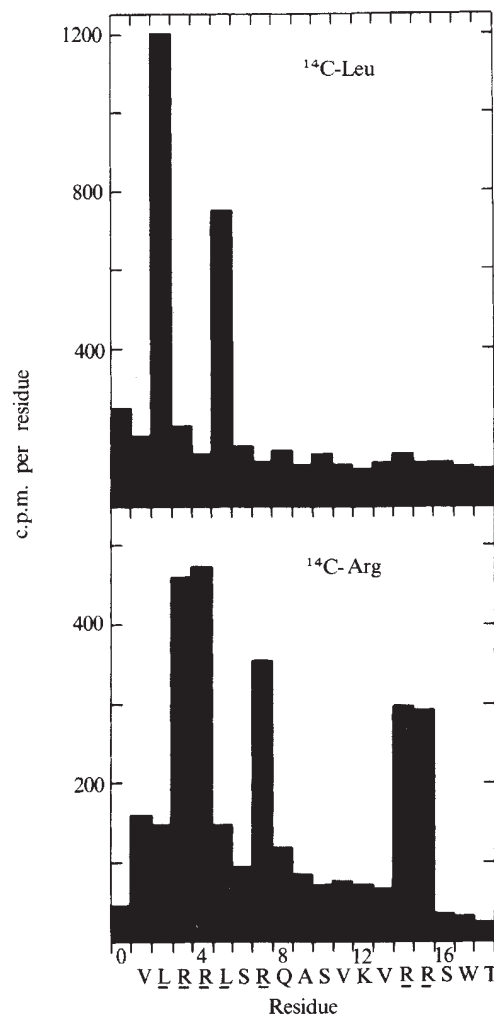


Fig. 3 Partial amino-terminal sequence determination of the SV40 agnogene product. Lysates of SV40-infected cells radiolabelled with ^{14}C -arginine or ^{14}C -leucine were enriched for the agnoproduct by affinity chromatography on a calf thymus DNA-cellulose column (see Fig. 5 legend), followed by electrophoresis on a 12.5% SDS-polyacrylamide gel. The position of the agnoproduct was identified by autoradiography of the dried gel and this region excised and eluted electrophoretically. The eluted samples were applied to a Beckman 890C automated protein sequencer together with 2 mg of apomyoglobin carrier protein and 3 mg of polybrene. About 6,500 c.p.m. of ^{14}C -arginine-labelled product and 7,400 c.p.m. of ^{14}C -leucine-labelled product were applied to the sequencer. The amount of radioactivity released after each Edman cycle is shown. The expected amino acid sequence of the SV40 agnoproduct amino-terminus is given in the single letter code at the bottom of the figure; identified residues are underlined. The initial yield, estimated from the apparent repetitive yield and the expected amino acid composition, was 76% for the arginine-labelled sample and slightly greater than 100% for the leucine-labelled sample.

of this sequence within the genome of the human papovavirus BKV^{16,17}, an evolutionary variant of SV40. About two-thirds of the amino acids in the putative BKV agnogene are identical to those of the SV40 protein (see Fig. 1*b*).

The location of the coding sequence for the SV40 agnogene within the leader of the major late mRNAs may suggest a role in transcription or processing of the late SV40 mRNA. In this regard, recent studies have indicated that an attenuator of late SV40 mRNAs is situated in the proximal late region¹⁸. It is possible that the agnoprotein is translated from an attenuated RNA and/or affects this process of attenuation.

Given the affinity of the agnoprotein for DNA and its basic nature, it is possible that, like histones, it might be associated with the viral nucleic acid core. Although preliminary experiments suggest that there is no detectable agnogene product in the SV40 virion (data not shown), this does not exclude the

possibility that the agnoprotein has a transient function in virion assembly.

We do not know which RNA species encode(s) the agnoprotein. Of the two major late SV40 polyadenylated transcripts (16S and 19S RNAs), the 16S mRNA is the more likely candidate because of its abundance and the fact that the agnogene coding sequence is found in its leader (5' exon). A considerable body of data has led to the supposition that 5'-terminal AUG codons are preferentially selected by eukaryotic translational

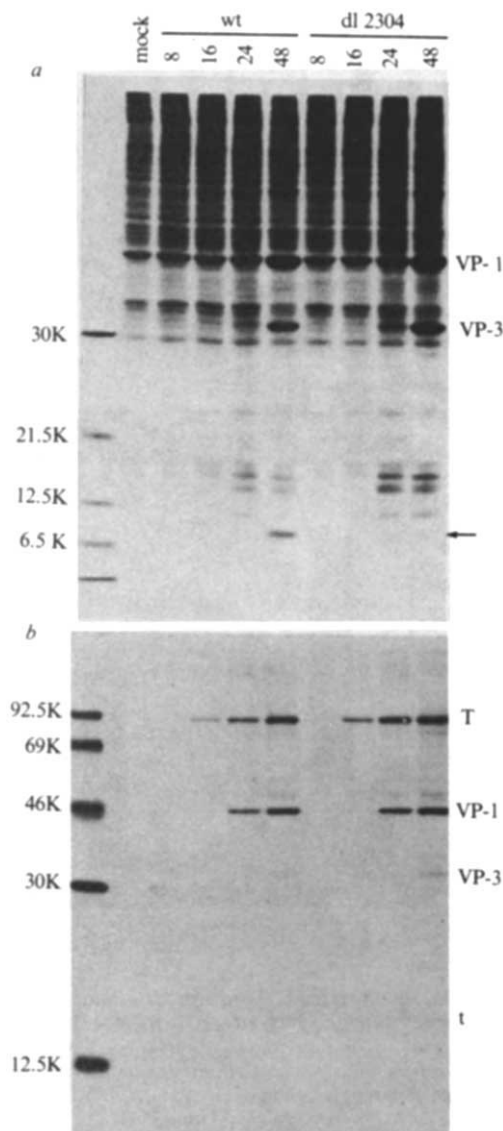


Fig. 4 Time course of the synthesis of the SV40 agnogene product. Cultures of AGMK cells were infected with either wt SV40 or dl 2304. At various times post-infection, cells were radiolabelled for 1 h with ¹⁴C-arginine and collected as described in Fig. 2 legend. Aliquots of the cell extracts were analysed either directly (*a*), or after immunoprecipitation with an SV40 anti-tumour serum (*b*) by electrophoresis on a 12.5% SDS-polyacrylamide gel, followed by fluorography and exposure for 12 h. The molecular weight markers used in *a* were the same as those described in Fig. 2 legend; those in *b* were phosphorylase *b* (92.5 K), bovine serum albumin (69 K), ovalbumin (46 K), carbonic anhydrase (30 K) and cytochrome *c* (12.5 K). The arrow indicates the position of the agnoprotein.

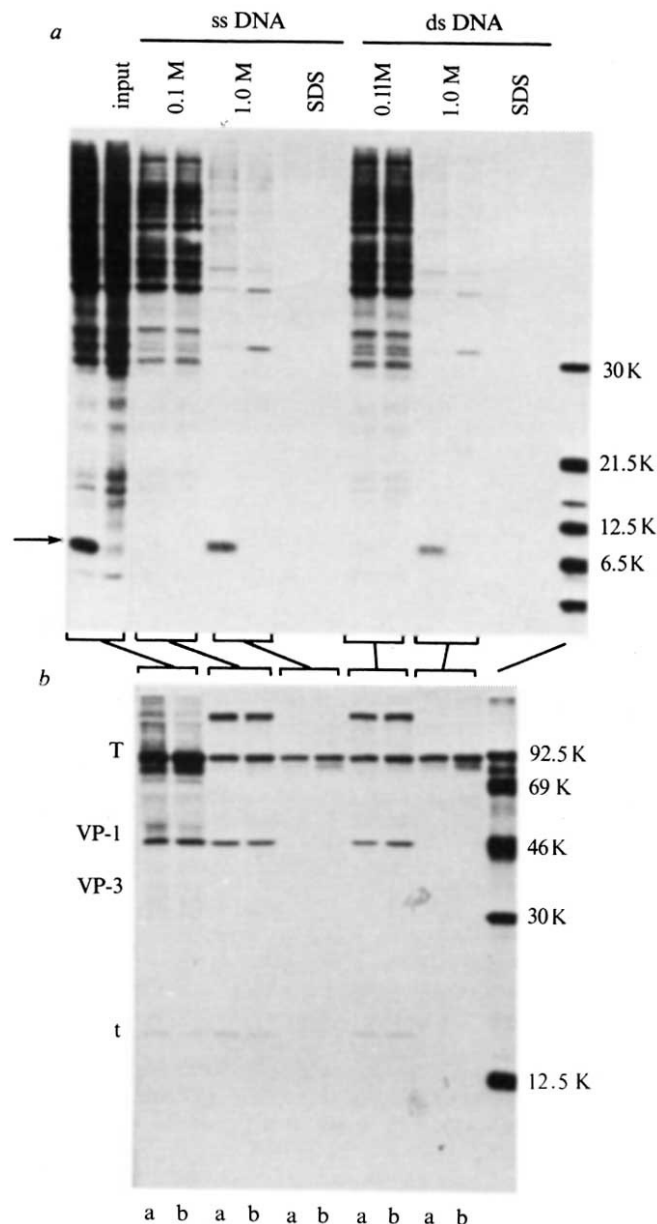


Fig. 5 Binding of the SV40 agnogene product to DNA. Cultures of AGMK cells, infected with either wt SV40 (*a*) or dl 2304 (*b*) at 10–20 PFU per cell, were labelled for 2 h with ¹⁴C-arginine at 48 h post-infection. Cells were collected and lysed in buffer B (Tris-buffered saline pH 8.0 containing 1% Triton X-100 and 25 μ g ml⁻¹ L-1-tosylamide-2-phenylethylchloromethylketone). The extracts were adjusted to pH 6.0 and loaded on to either denatured (ss) or native (ds) calf thymus DNA-cellulose columns, previously equilibrated with 5 mM sodium phosphate, pH 6.0/0.1 M NaCl/0.5% Nonidet P-40/10% glycerol. The columns were washed with the same buffer and the bound fractions were stepwise eluted, first with the same buffer containing 1 M NaCl then with buffer containing 2% SDS and 5% 2-mercaptoethanol. The various fractions were analysed either directly (*a*) or after immunoprecipitation with anti-tumour serum (*b*) in 12.5% SDS-polyacrylamide gels. The molecular weight markers were the same as those described in Fig. 4 legend. The arrow indicates the position of the agnoprotein.

systems (see ref. 19). But until now, the late 16S RNA had been considered to be an exception as the agnogene AUG precedes the AUG initiator codon which was known to be used for the translation of VP1. If the 16S mRNA is the translational template for the agnoprotein, the 5'-terminal AUG is a translational initiator. However, the late 16S SV40 RNA would then represent a unique polycistronic eukaryotic mRNA which can encode both the agnoprotein and VP1.

The absence of methionine residues from the agnoprotein as well as its rapid turnover rate may explain why it has not previously been detected. We have recently learned that other investigators have found a similar polypeptide in SV40-infected cells (P. Southern and P. Berg, J. Mertz, R. Margolsky and D. Nathans, personal communications).

This work was supported in part by the US Department of Energy. We thank Carol Prives for valuable suggestions.

Received 15 December 1980; accepted 5 March 1981.

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Mutagenesis *in vitro* by depurination of Φ X174 DNA

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Depurination, the spontaneous release of purine bases from DNA, occurs by the breakage of the N-glycosylic bond between the purine base and the deoxyribose moiety. The resulting apurinic site is relatively stable, with an *in vitro* half life of up to several days¹. The rate constant of depurination *in vitro* has been estimated to be 1.8×10^{-9} per min (ref. 2). This corresponds to the formation of 20,000 apurinic sites per mammalian cell per day. In addition, much of the damage to DNA by chemical carcinogens occurs through covalent binding to the N-3 and N-7 positions on the purine bases^{3,4}, enhancing the rate of depurination of that base by three to four orders of magnitude^{5–7}. The fact that cells have multiple pathways for removing apurinic sites^{8,9} and even a mechanism for reinserting purines¹⁰ suggests that the presence of apurinic sites may be highly detrimental. Apurinic sites in DNA have been postulated as potential mutagenic lesions¹¹, although there has been little experimental support for this concept. We have previously demonstrated that depurination of synthetic polynucleotide templates leads to increased mis-incorporation of non-complementary nucleotides^{12,13}. We now present evidence that a DNA polymerase can copy past apurinic sites on natural, biologically active DNA and further, that this leads to increased mutagenesis.

Table 1 *E. coli* DNA polymerase I copies past apurinic sites on Φ X174 DNA

Fragment strand	Neutral sucrose sedimentation coefficient	Alkaline sucrose sedimentation coefficient
Non-depurinated Z-7 fragment		
Template [³ H]	5.7	4.4
Product [³² P]	5.7	4.4
Depurinated Z-8 fragment		
Template [³ H]	5.3	2.8
Product [³² P]	5.3	4.0

³H-labelled Φ X174 single-strand *am3* DNA was brought to a pH of 2.75 with HCl and incubated at 55 °C for 25 min to produce 2% depurination. The non-depurinated sample was not incubated. The samples were neutralized to pH 7.8 with 1 M Tris-HCl (pH 8.1). A fivefold molar excess of *Hae*III restriction fragment Z-1 was hybridized to the Φ X174 DNA template by first heating at 90 °C for 5 min, followed by 5 min at 0 °C and finally 30 min at 65 °C. *In vitro* synthesis was performed at 37 °C for 30 min in a reaction containing 20 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 100 μ M dATP, dCTP, dGTP and [α -³²P]dTTP (800 c.p.m. pmol⁻¹), 5 mM MgCl₂ and *E. coli* Pol I at a 25:1 molar excess to template DNA molecules. The extent of synthesis, reported as a calculated average number of nucleotides added per template molecule, was 1,370 and 4,880 for the depurinated and non-depurinated samples, respectively. The DNA was precipitated with ethanol, washed five times with 70% ethanol to remove excess salt, resuspended in 10 mM Tris-HCl (pH 8.0) and digested with *Hae*III restriction endonuclease as described previously¹⁵. The resulting restriction fragments were separated by electrophoresis on polyacrylamide gels¹⁵, electroeluted from the gels in buffer E, ethanol precipitated and resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. Analysis of individual fragments by neutral and alkaline sucrose gradients was performed as previously described¹⁵, using linear 5–20% gradients. Centrifugation at 4 °C in an SW 50.1 rotor was for 16 h at 45,000 r.p.m. Parallel gradients contained Z-5, Z-7, Z-8 and Z-10 fragments as markers.

To determine whether a DNA polymerase can copy past apurinic sites on natural DNA we characterized the product of the reaction using as a template single-stranded circular Φ X174 DNA containing a known number of apurinic sites. The experiment was performed as follows. A template strand of single-stranded ³H-labelled Φ X174 *am3* DNA was depurinated by incubation at 55 °C at a pH of 2.75 and a single *Hae*III restriction fragment was annealed to the template to function as a fixed primer for copying with *Escherichia coli* DNA polymerase I (Pol I) and [α -³²P]-labelled deoxynucleoside triphosphates (dNTPs). The product was digested with restriction endonuclease *Hae*III and the resulting restriction fragments separated by gel electrophoresis and recovered by electroelution; individual restriction fragments were analysed by sedimentation on neutral and alkaline sucrose gradients. As in our previous studies with synthetic polynucleotides¹³, depurination inhibits DNA synthesis. However, three findings suggest that the enzyme copied past apurinic sites in the DNA. First, with a Φ X template (5,386 nucleotides long) containing ~50 apurinic sites, the average number of nucleotides incorporated per template was 1,370, suggesting that DNA synthesis proceeded past at least 10–15 apurinic sites (Table 1 legend). (This assumes that synthesis is synchronous and depurination is random.) Second, in this experiment synthesis was initiated with *Hae*III restriction fragment Z-1. As the fragments are synthesized in the order Z-1, Z-4, Z-8 (ref. 14), recovery of label in Z-8 indicates that synthesis proceeded past Z-4 (603 nucleotides) and Z-8 (194 nucleotides), together containing an estimated eight apurinic sites.

The third and most direct evidence that DNA polymerases copy past apurinic sites is obtained from sucrose gradient analysis of individual restriction fragments (Table 1) and takes advantage of the fact that apurinic sites in DNA are alkali labile. In neutral sucrose, both the ³H-labelled template strand and the ³²P-labelled product strand of the isolated restriction fragment

Table 2 Depurination decreases the accuracy of DNA synthesis *in vitro*

Apurinic sites per molecule	Nucleotides added per template	Reversion frequency ($\times 10^{-6}$)	Error rate
Experiment 1: Mg^{2+} , $10\times$ [dCTP]			
0	2,470	2.10	1/92,900
1.1	2,280	13.6	1/14,300
1.4	1,710	19.5	1/10,000
3.2	1,150	28.6	1/6,820
5.8	955	44.7	1/4,360
Experiment 2: Mn^{2+} , equal [dNTPs]			
0	979	51.6	1/3,780
1.1	924	69.3	1/2,810
1.4	887	80.3	1/2,430
3.2	708	158	1/1,230
5.8	676	264	1/739

Φ X174 *am3* DNA was depurinated by heating at 70 °C, pH 5.0. Hybridization of *Hae*III restriction fragment Z-8 and synthesis with Pol I were as described in Table 1 legend. In experiment 1, the dCTP concentration was 1 mM; in experiment 2, 0.5 mM Mn^{2+} was substituted for Mg^{2+} . The transfection assay for determination of reversion frequencies and the method for calculating the error rate are as described previously^{15,16}. The background reversion frequency was 2.68×10^{-6} and 1.90×10^{-6} for experiments 1 and 2, respectively, and were subtracted from the values shown in the Table. In these experiments, the reversion frequency of uncopied DNA is the same for both depurinated and non-depurinated DNA.

show coincident peaks with either the depurinated or non-depurinated samples (Table 1). The sedimentation coefficients correspond to the length of the individual fragment under study. In alkaline sucrose, the 3H - and ^{32}P -labelled strands obtained with a non-depurinated template remain coincident but with a sedimentation coefficient corresponding to that of a single-strand fragment. With the depurinated sample, the ^{32}P -labelled product strand sediments to the position of an intact single-strand fragment. In contrast, the 3H -labelled template strand, which should contain alkali labile apurinic sites, sediments more slowly. This change in the sedimentation coefficient from 4.0 to 2.8 predicts two to three sites of cleavage, in agreement with our estimate of two sites in the Z-8 fragment based on 2% depurination of the Φ X template.

The mutagenic potential of apurinic sites in DNA was assessed using the recently developed Φ X assay¹⁴⁻¹⁶. The accuracy of *in vitro* DNA synthesis is quantitated by measuring the reversion frequency of an amber mutation to wild type in a transfection assay using *E. coli* spheroplasts. With Φ X174 *am3* DNA, reversion to wild type has been shown to occur by at least two different substitutions¹⁶. The TAG amber codon may be converted to either a TGG or a TTG codon. In both cases, the substitution occurs opposite the adenine in the template, a potential site for depurination. Table 2 shows the effects of depurination of Φ X174 *am3* DNA on the reversion frequency.

Because Pol I is highly accurate in Mg^{2+} -activated reactions, changes in accuracy were measured in conditions of DNA synthesis using a selective 10-fold increase in the concentration of dCTP (ref. 16), so as to obtain a baseline error rate for the non-depurinated control. With progressively depurinated templates, the reversion frequency increases from 2.1×10^{-6} with no depurination to 44.7×10^{-6} with 5.8 apurinic sites per molecule (Table 2, experiment 1). This corresponds to a change in the error rate from 1/92,900 to 1/4,360. Substitution of Mn^{2+} for Mg^{2+} results in a 100-fold increase in mis-incorporation, permitting measurement of fidelity with unbiased nucleotide pools^{15,16}. With Mn^{2+} , the reversion frequency increases from 51.6×10^{-6} with a non-depurinated template to 264×10^{-6} with a template containing 5.8 apurinic sites per molecule, corresponding to a change in the error rate from 1/3,780 to 1/739.

Evidence that the enhancement of mutagenesis *in vitro* by depurination is mediated by substitution at the *am3* locus is suggested by the specificity observed when the relative concentrations of individual nucleotides are biased. As previously reported for Pol I with non-depurinated templates in Mg^{2+} , the reversion frequency is increased in proportion to the relative concentration of dCTP in the reaction¹⁶. The response is linear, with a 10-fold increase in dCTP concentration giving a 10-fold increase in reversion frequency. In contrast, a 10-fold increase in dATP concentration results in only a slight increase in reversion frequency, suggesting that mis-incorporation of A (in Mg^{2+}) is normally a less frequent event at the *am3* locus. With depurinated templates, dCTP is again mutagenic, as expected for substitution at *am3*. In addition, dATP is now highly mutagenic (Table 3), suggesting that random substitution might be occurring opposite a depurinated site in the *am3* codon.

In our depurination conditions, the only other known lesion produced capable of reverting the amber mutation would be deamination of adenine. However, depurination is more than 10^5 times more frequent than deamination of adenine⁹. More importantly, as shown in Table 3, alkali treatment of the depurinated DNA eliminates almost all the enhanced mutagenesis resulting from depurination. Hydrolysis of the apurinic sites by alkali reduces the enhanced reversion frequencies due to depurination by 94–100%, suggesting that the mutagenic effects observed are indeed due to the presence of apurinic sites.

Even though apurinic sites on DNA have been postulated as potential mutagenic lesions¹¹, there has been little experimental support for this concept. The predominant biological effect of depurination previously recognized was inactivation of DNA *in vivo*^{17,18}. However, depurination inhibits DNA synthesis and enhances mis-incorporation when copying either synthetic polynucleotides^{12,13} or natural DNA *in vitro* (this study). As there are multiple pathways for the repair of apurinic sites *in vitro*⁸⁻¹⁰, the likelihood of mis-coding at apurinic sites might be considerably less than that resulting from copying stable small modifications of bases such as *O*⁶-methylguanine¹⁹. However, larger adducts such as those resulting from aromatic polycyclic

Table 3 Alkali treatment reverses the mutagenicity of depurination

Conditions	Non-depurinated DNA		Depurinated DNA		Alkali-treated depurinated DNA		
	Reversion frequency ($\times 10^{-6}$)	Error rate	Reversion frequency ($\times 10^{-6}$)	Error rate	Reversion frequency ($\times 10^{-6}$)	Error rate	% Recovery
Equal [dNTPs]	—	1/680,000*	5.25	1/37,100	0.60	1/325,000	93.7
$10\times$ [dCTP]	2.38	1/81,900	22.7	1/8,590	3.45	1/56,500	94.7
$50\times$ [dCTP]	15.9	1/12,300	53.6	1/3,640	15.8	1/12,300	100
$50\times$ [dATP]	2.53	1/77,100	58.4	1/3,340	6.05	1/32,200	93.7

Depurination of Φ X174 *am3* DNA was as described in Table 2 legend. Alkali treatment was performed by incubation at 37 °C for 30 min in the presence of 0.2 M NaOH followed by neutralization with 1 M HCl. Hybridization of *Hae*III restriction fragment Z-5 and synthesis conditions were as described in Table 1 legend. Nucleotide pool biases are as indicated with equal dNTPs being 50 μ M. The transfection assay and determination of reversion frequency and error rate are as previously described^{15,16}. The background reversion frequency, which has been subtracted from all values listed in the Table, was 1.63×10^{-6} . The survival of the depurinated DNA was 11% for uncopied DNA, representing an average of 2.2 apurinic sites per molecule²².

* Taken from ref. 14, extrapolated from dCTP pool bias experiments.

hydrocarbons block DNA replication *in vitro*²⁰. Moreover, blockage of DNA replication *in vivo* has been postulated to induce the expression of SOS functions²¹. In fact, we have been able to observe an enhanced reversion frequency when uncopied depurinated DNA is transfected in spheroplasts derived from bacteria in which error-prone DNA repair is induced by UV light²². Furthermore, adducts formed with aromatic hydrocarbons, particularly those involving the N-7 of guanine and the N-3 of adenine enhance depurination⁵⁻⁷. For example, 20% of the binding of the activated carcinogen, benzo[a]pyrene diol epoxide is on the N-7 of guanine²³. Also, the major adduct formed with aflatoxin, a known mutagen²⁴ and one of the most potent carcinogens, is on the N-7 of guanine²⁵. Finally, Drinkwater *et al.*²⁶ have recently reported a strong positive qualitative correlation between the ability of a series of carcinogens to depurinate DNA and their missense mutagenic activity. Although this correlation is not quantitative, it supports the idea that at least some of the mutagenicity associated with known carcinogens may result from depurination.

Received 8 December 1980; accepted 16 March 1981.

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Sym plasmid of *Rhizobium trifolii* expressed in different rhizobial species and *Agrobacterium tumefaciens*

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Bacteria of the genus *Rhizobium* fix nitrogen in symbiotic association with legumes such as clover, pea and bean. If the efficiency of this plant-bacterium relationship is to be improved or the host range of the rhizobia extended, the genes involved in the symbiosis must first be identified and localized. Accumulating genetic and biochemical data support the proposal that genes on a large plasmid¹ are involved in root nodulation, in analogy with the Ti genes of *Agrobacterium tumefaciens*, which is closely related to the fast-growing species of *Rhizobium*²⁻⁴ (for reviews, see refs 5, 6). Although most of the data concern *Rhizobium leguminosarum*⁷⁻¹¹, some deal with *Rhizobium trifolii*¹²⁻¹⁴, which associates with clover. We report here that one particular plasmid in *R. trifolii*—which we call Sym(biosis) plasmid—not only determines host specificity, but also controls several, if not most, other steps leading to root nodule formation and nitrogen fixation on clover roots.

To provide a selectable marker, we tagged the *R. trifolii* plasmid with transposon Tn5, which confers kanamycin resistance (Km^r) on its host cell. *Escherichia coli* JB1830 (ref. 15), which carries an R::Mu::Tn5 suicide plasmid coding for Gm^rKm^rSm^rSp^r, was crossed with a Rif^r *R. trifolii* (LPR5001) and we then selected for Km^r *R. trifolii* derivatives. Several Km^rRif^r (but Gm^rSm^rSp^r) transconjugants—which arose at a frequency of 4×10^{-7} per recipient—were used as possible donors in further crosses with a Str^r *R. trifolii* strain (LPR5020). Out of 10 possible Km^r donors, five transferred Km^r to LPR5020 at a frequency of 10^{-4} – 10^{-6} per recipient. To verify that no spontaneously Str^r donors were present among the Km^rStr^r transconjugants, these were checked for and found to be Rif^r.

To determine whether genes on the self-transmissible *R. trifolii* plasmid (referred to here as pRtr5a) that was marked with Tn5 were involved in symbiotic nitrogen fixation, plasmid-cured derivatives were isolated as described previously¹⁴. After incubation of the *R. trifolii* strains for 2 days at 37 °C, we found among the surviving bacteria—most of the bacteria are killed by this heat treatment—several Km^r derivatives. These were Nod⁻, and by all other criteria were identical to the parental strains

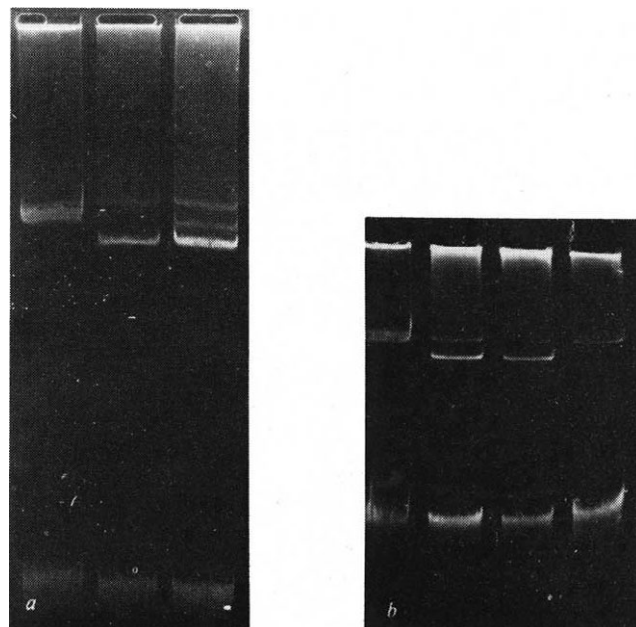


Fig. 1 Agarose gel electrophoresis of plasmid DNA isolated and run according to Casse *et al.*²². DNA was prepared from (from left to right): a: *R. trifolii* LPR5035, *R. leguminosarum* LPR1902, *R. leguminosarum* LPR1902 (pRtr5a); b: *R. trifolii* LPR5035, *R. meliloti* LPR2136 (pRtr5a), *A. tumefaciens* LBA288 (pRtr5a).

Table 1 Properties of a Sym plasmid-cured *R. trifolii* strain (LPR5039) compared with the parental strain (LPR5035)

	<i>R. trifolii</i> LPR5035	<i>R. trifolii</i> LPR5039
Resistance to streptomycin (250 µg ml ⁻¹)	+	+
Growth on NB or LC medium	—	—
Sensitivity to phages LPB1, 51, 52	+	+
Uptake of Congo red from medium	—	—
Nodulation of clover seedlings*	+	—
Resistance to kanamycin (50 µg ml ⁻¹)	+	—
Presence of pRtr5a::Tn5	+	—

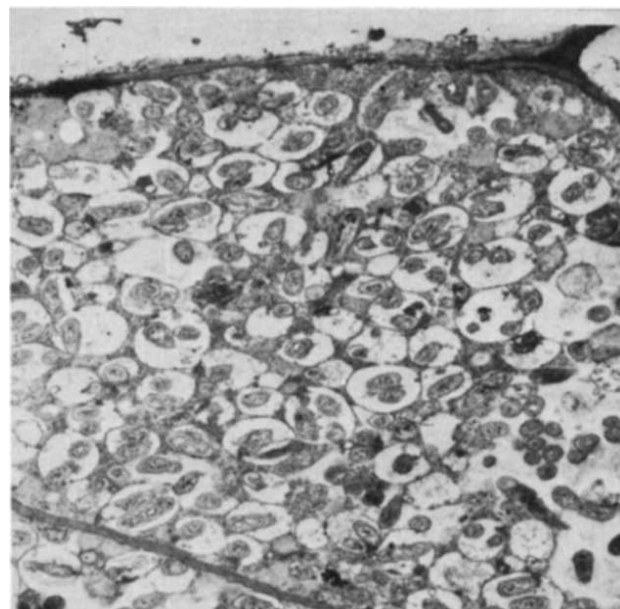
**Trifolium pratense*.

Table 2 Properties of *R. leguminosarum* strains before and after acquisition of the *R. trifolii* Sym plasmid

Recipient strain	Nodulation of <i>Trifolium pratense</i>		N fixation on <i>T. pratense</i>		Nodulation of <i>Vicia hirsuta</i>		N fixation on <i>V. hirsuta</i>	
	R	T	R	T	R	T	R	T
LPR1105	—	+	—	+	+	+	+	+
LPR1902	—	+	—	+	+	+	+	+
LPR1802	—	+	—	+	—	—	—	—
LPR1120	—	+	—	+	+	+	—	—

R, property of the recipient; T, property of the pRtr5a⁺ transconjugant; LPR1105 = RCR1001 Rif^r; LPR1120 = RCR1017 Rif^r; LPR1802 = RCR1018 Rif^r; LPR1902 = RCR1039 Rif^r.

(Table 1). We showed biochemically that these strains had lost a plasmid of molecular weight 180×10^6 and that the Nod[−]Km^s phenotype was not due to a deletion of part of the plasmid (not shown). This suggested that we had indeed marked a symbiosis-controlling (Sym) plasmid with Tn5. To confirm this and to exclude the possibility that secondary mutations had been introduced by the heat treatment, the plasmid was re-introduced into the Km^sNod[−] strains by conjugation. Acquisition of pRtr5a rendered these recipients Nod⁺Fix⁺, showing that this self-transmissible plasmid in *R. trifolii* controls nodulation. The

**Fig. 2** Efficiency of clover plant growth in N-free medium after inoculation with: left, *R. leguminosarum* (pRtr5a::Tn5); centre, *R. trifolii* RCR5; right, *A. tumefaciens* (pRtr5a::Tn5).**Fig. 3** Electron microscopic view inside a clover plant cell filled with agrobacteria surrounded by a membrane envelope. $\times 3,200$.

frequency of transfer of pRtr5a::Tn5 to the plasmid-cured *R. trifolii* strains was as high as to the corresponding plasmid-containing strains, indicating the lack of entry exclusion functions on this plasmid. After crossing, pRtr5a⁺ transconjugant colonies derived from the plasmid-cured *R. trifolii* strains developed more rapidly than those derived from the corresponding plasmid-containing strains. This may have been due to incompatibility between incoming and resident Sym plasmid in the latter case.

The expression of pRtr5a was studied in several *R. leguminosarum* strains. As Table 2 shows, transfer to *R. leguminosarum*, which is closely related to *R. trifolii*²⁻⁴ but nodulates peas and vetches, resulted in strains capable of nodulating not only their normal symbiotic partners but also clover.

Table 3 Properties of an *A. tumefaciens* transconjugant carrying pRtr5a compared with those of the parental strains

	<i>R. trifolii</i> ‡ LPR5035	<i>A. tumefaciens</i> ‡ LBA288 (pRtr5a::Tn5)	<i>A. tumefaciens</i> ‡ LBA288
Resistance to streptomycin*	+	—	—
Resistance to rifampicin*	—	+	+
Sensitivity to phage:			
LPB 1, 51, 52	+	—	—
LPB 84	—	+	+
Growth on NB or LC medium	—	+	+
Growth on erythritol medium	+	—	—
Uptake of Congo red from medium	—	+	+
Nodulation of clover seedlings†	+	+	—
N fixation in root nodules	+	—	—
Tumour induction on plants	—	—	—

* Streptomycin at $250 \mu\text{g ml}^{-1}$; rifampicin at $20 \mu\text{g ml}^{-1}$.

† *Trifolium pratense*.

‡ LPR5035 = RCR5 Str^r (pRtr5a::Tn5); LBA288 = C58-C9 Rif^rNal^r.

This suggests that the plasmid must control host specificity. The plasmid profile of a transconjugant is compared with that of the parental strains (Fig. 1). As far as Fig. 1 shows, no *R. leguminosarum* plasmid was lost from the recipients after the introduction of pRtr5a. Introduction of the plasmid into a Nod⁻ *R. leguminosarum* mutant (LPR1802) rendered this strain Nod⁺Fix⁺ on clover, but it remained Nod⁻ on peas and vetches. Plasmid pRtr5a apparently does not abolish the inability of LPR1802 to nodulate peas and vetches. Introduction of the plasmid into a Nod⁺Fix⁻ *R. leguminosarum* strain (LPR1120), which lacks at least a segment of the *nif* structural genes (R. K. Prakash, personal communication), rendered this strain Nod⁺ and Fix⁺ on clovers, showing that the Sym plasmid carries at least some of the *nif* structural genes. The latter finding was confirmed biochemically¹⁶. These results support the proposal that bacteria of the species *R. trifolii* and *R. leguminosarum* are identical except for their plasmid content⁷.

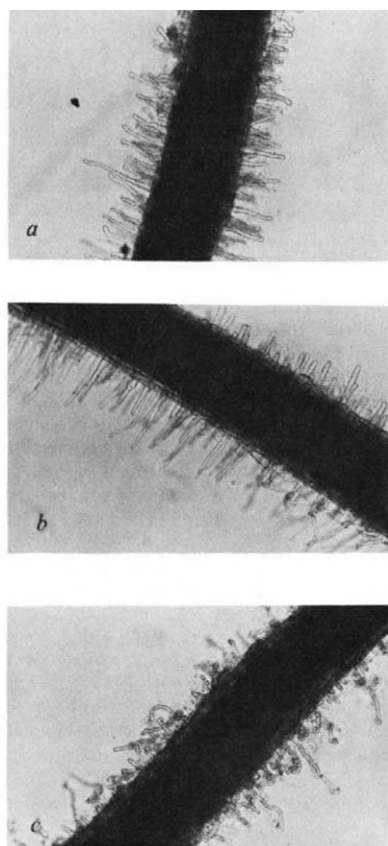


Fig. 4 Induction of root hair curling on clover roots. Clover seedlings on Jensen agar medium²³ were inoculated with bacteria, and after 5 days examined with the light microscope. *a*, No bacterial inoculation; *b*, inoculation with *A. tumefaciens* LBA288; *c*, inoculation with *A. tumefaciens* LBA288 (pRtr5a). $\times 180$.

As we have found that the *A. tumefaciens* Ti plasmid can be transferred to *R. trifolii*, where it is stably maintained and expressed^{5,17,18}, we wished to see whether the *R. trifolii* Sym plasmid could be transferred to *A. tumefaciens* and whether its genes would be expressed there. To avoid interference with Ti plasmid-encoded functions, the Sym plasmid was introduced into a Ti plasmid-cured derivative of *A. tumefaciens* strain C58 (LBA288). Purified transconjugants seemed to be almost identical to the recipient LBA288 (Table 3), except that they could weakly induce white non-nitrogen-fixing root nodules on clover roots. The nodules appeared after a longer period of incubation (4–5 weeks) than did the nodules induced by *R. trifolii* (1 week). The bacteria recovered from inside the root nodules were

identical to those used for inoculation, excluding the possibility that nodules had been due to contaminating rhizobia. Because of a lack of nitrogen, the plants infected with *A. tumefaciens* transconjugants did deteriorate (Fig. 2). Squashes of the *A. tumefaciens*-induced nodules examined under the light microscope revealed no typical bacteroids, such as the γ -shaped structures that were numerous in squashes of nodules induced by *R. trifolii*. Electron microscopy, however, showed that the nodules induced by *A. tumefaciens* transconjugants were true root nodules. Infection threads filled with bacteria were clearly visible. Furthermore, bacteria surrounded by a membrane were prominent inside clover plant cells, showing that the agrobacteria had been released from the infection thread into the plant cell (Fig. 3).

An essential step early in root nodule formation is the induction of a marked curling of the root hairs by the rhizobia¹⁹. Certain Nod⁻ mutants cannot induce this root hair curling²⁰. As our *A. tumefaciens* transconjugants with the *R. trifolii* Sym plasmid seemed to induce only sparse root nodules, we tested whether they could induce such curling. As Fig. 4 shows, there was a marked curling of clover root hairs after inoculation with the Sym plasmid harbouring *A. tumefaciens* transconjugants, but not after inoculation with *A. tumefaciens* parental strain LBA288 (Fig. 4), thus indicating that induction of root hair curling is an event controlled by the *R. trifolii* Sym plasmid. In agreement with this conclusion, *R. trifolii* strains lost the ability to induce curling of clover root hairs when cured of their Sym plasmid.

Our observation that introduction of the *R. trifolii* Sym plasmid into *A. tumefaciens* results in bacteria that, in principle, can bring about root nodulation (root hair curling, host recognition, infection thread initiation, release of membrane enclosed bacteria in the plant cell) shows that *R. trifolii* Sym plasmid can be expressed in this more distant bacterium. It is unclear why only sparse nodules, which do not fix nitrogen, appear late after infection, but the absence of typical γ -shaped bacteroids in nodules induced by *A. tumefaciens* might explain why no nitrogen is fixed in the nodules. Similar results were obtained by E. Pees and C. Wiffelman (personal communication) with root nodules induced on vetches by *A. tumefaciens* harbouring an *R. leguminosarum* Sym plasmid. These Sym plasmids are clearly different from a very large *R. trifolii* plasmid that was recently introduced into *A. tumefaciens* by mobilization with RP4 and which conferred the ability to fix nitrogen, but not the ability to induce root nodules on its new host (ref. 13 and J. Stanley, personal communication).

We thank Dr C. Wiffelman and M. P. Nuti for helpful discussions and Cebeco-Handelsraad for providing clover seeds. During preparation of this manuscript it was shown that root attachment is controlled by an *R. trifolii* plasmid²¹.

Received 28 November 1980; accepted 16 March 1981.

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Recombinant plasmids carrying nitrogen fixation genes from *Rhizobium japonicum*

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The bacterium *Rhizobium japonicum*, which fixes atmospheric nitrogen in symbiosis with soybean roots is a member of the so-called 'slow-growing' group of *Rhizobium*, which implies that its *nif* genes are depressed in free-living, microaerobic growth conditions. This makes it a potentially useful organism for studies of the molecular biology of the regulation of symbiotic *nif* genes^{1,2}. Although classical genetic experiments with this species are not well advanced, we decided to use recombinant DNA techniques to isolate and characterize its *nif* genes. Comparative studies on the biochemistry³, immunology^{2,4} and genetics⁵ of nitrogenase from different N₂-fixing microorganisms suggested a fairly strong DNA sequence homology for the nitrogenase structural genes of the species investigated. We have therefore used the cloned nitrogenase structural genes from a free-living, N₂-fixing bacterium, *Klebsiella pneumoniae*⁶, as hybridization probes⁵ to find homologous DNA regions in a clone bank of DNA fragments of *R. japonicum*. We report here that the *nif* homology region includes two of the nitrogenase genes, *nifH* and *nifD*.

Using the restriction enzyme *Hind*III we have constructed a bank of 3,325 *Escherichia coli* colonies containing cloned fragments of the total genome of *R. japonicum* strain 110 (ref. 7). The cloning vector was pBR322 (ref. 8), which has a single *Hind*III site within the genes coding for tetracycline resistance. The *K. pneumoniae* DNA probe used for hybridization came from plasmid pSA30 (ref. 6), and represents an *Eco*RI fragment containing almost exclusively the nitrogenase structural genes, *nifK*, *nifD* and *nifH* (Fig. 1). This *Klebsiella* DNA fragment was purified by preparative agarose gel electrophoresis and nick-translated⁹ in the presence of ³²P-thymidine-triphosphate to high specific radioactivity (10⁸ c.p.m. per µg DNA). Colonies of the colony bank were grown on nitrocellulose filters to a size of ~2–3 mm. The filters were then layered on to LB agar¹⁶ containing 100 µg ml⁻¹ chloramphenicol (for 24 h) to facilitate amplification of the recombinant plasmids within the cells.

The ³²P-labelled *Klebsiella nif* DNA was used to detect *R. japonicum nif* genes by colony hybridization¹⁰; of the 3,325 colonies screened, 21 contained DNA that clearly hybridized. The recombinant plasmids were isolated from all 21 strains. Each plasmid had a *Hind*III DNA insert of molecular weight (MW) 4.85 × 10⁶; five of these had a second insert and one had two further inserts (not shown). Southern blotting experiments¹¹ showed that in all 21 cases it was the 4.85 × 10⁶ MW fragment that hybridized to *Klebsiella nifKDH* DNA. Plasmid pRJ676 from colony 676, a representative of the first group of recombinant plasmids, was studied in more detail.

First, a detailed restriction map of pRJ676 was established, using the restriction enzymes *Hind*III, *Eco*RI, *Pst*I, *Bam*HI and *Sal*I (Fig. 1). Single digests as well as double digests in every possible combination were done. The molecular weights of the fragments were determined using molecular weight markers (digested phage λ DNA), and we used the known restriction sites on pBR322 (ref. 12) to determine the molecular weights of those fragments adjacent to the vector. Precise location of the restriction sites was made possible by isolation and analysis of nine individual subclones from the various fragments of the 4.85 × 10⁶ MW pRJ676 insert (Fig. 1). The vectors used for recloning the smaller DNA pieces are described in Fig. 1 legend. From this restriction map it was possible to determine the

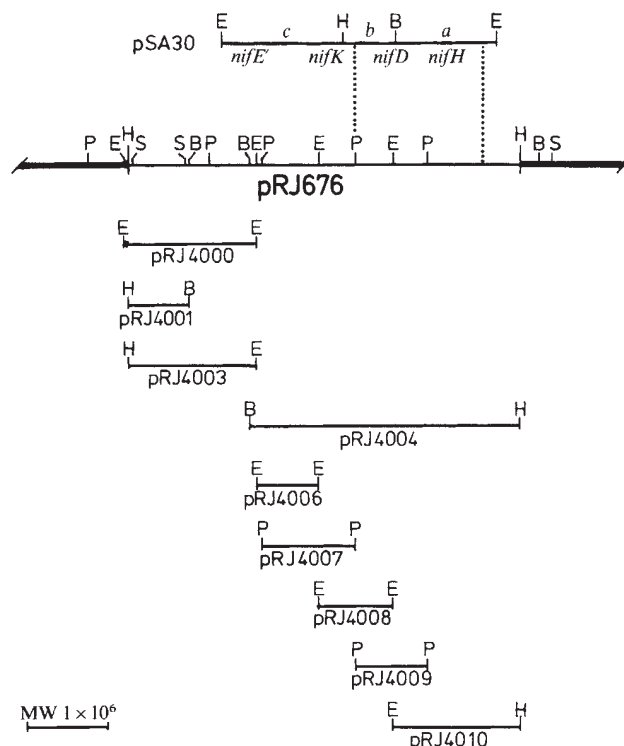


Fig. 1 Recombinant plasmids used in this study. For all plasmids, except pRJ676, only the cloned DNA inserts are shown. Restriction sites are: E, *Eco*RI; B, *Bam*HI; H, *Hind*III; P, *Pst*I; S, *Sal*I. The scale bar represents a length of DNA of MW 1 × 10⁶. The *Eco*RI fragment of pSA30 (ref. 6) is shown together with the approximate location of the *K. pneumoniae* nitrogenase structural genes, *nifK*, *nifD* and *nifH*, and with the subfragments *a*, *b* and *c*, which were used for the hybridization experiment in Fig. 2B. pRJ676 is one representative of the recombinant plasmids carrying the *nif* homologous region of *R. japonicum*. The cloning vector pBR322 (ref. 8) is indicated by a heavy line, and is arbitrarily split into two halves. The dotted lines enclose the approximate region of DNA homology between the *K. pneumoniae* and *R. japonicum nif* DNAs. A series of restriction fragments were cloned into the following cloning vectors (in parentheses): pRJ4006 (pACYC184, ref. 14); pRJ4000, pRJ4008 (pUR2, ref. 15); all others (pBR322, ref. 8). In these subcloning experiments pRJ676 and the respective cloning vectors were digested with one single or two different restriction enzymes, and the resulting fragments ligated with DNA ligase. The rejoined DNA was transformed into *E. coli* strain HB101 (when pBR322 or pACYC184 were the vectors) or into strain 79-02 (when pUR2 was the vector). The transformants were tested for the presence (or absence, due to insertion) of the vector's antibiotic resistance phenotype(s). In the case of vector pUR2, clones with inserted foreign DNA could be visualized directly as white colonies on Xgal plates¹⁵. Plasmid DNA of presumptive subclones was then isolated and subjected to DNA restriction enzyme analysis.

approximate location of the DNA region within pRJ676 that exhibits homology with *K. pneumoniae nifKDH* DNA. These experiments were done by transferring restriction patterns of pRJ676 from the agarose gel on to nitrocellulose filters (Southern blotting¹¹), and hybridizing them with the nick-translated *Eco*RI insert of pSA30. A few examples are shown in Fig. 2A, for which we used the *Hind*III, *Bam*HI, *Hind*III/*Bam*HI and *Eco*RI restriction patterns. The hybridizing band could be clearly identified by direct comparison with the stained bands on the gels.

Next we asked which of the structural genes of the *K. pneumoniae* nitrogenase complex—*nifH*, *nifD* or *nifK*—are homologous to *R. japonicum nif* DNA. To find the answer we purified DNA subfragments of the *Klebsiella nifKDH* region (designated fragments *a*, *b* and *c* in Fig. 1) as molecular hybridization probes for the *Eco*RI pattern of pRJ676. Figure 2B

shows that subfragment *a* (containing *nifH* and part of *nifD*) hybridizes to the large *EcoRI* fragment of pRJ676, and subfragment *b* (containing part of *nifD* and part of *nifK*) hybridizes to one of the two smaller *EcoRI* fragments of pRJ676. Subfragment *c* (containing the larger part of *nifK* and part of the genes downstream of the *nif* cluster) seems to have no DNA homology with any region of pRJ676. Thus, interspecies hybridization seems to be based mainly on *nifH* and *nifD* homologies. However, these experiments do not completely exclude the possibility that at least part of *nifK* DNA also hybridized to *R. japonicum* DNA.

Several control experiments confirmed these results: (1) the cloned *R. japonicum nif* fragment of MW 4.85×10^6 was itself purified and used as a hybridization probe on the *EcoRI/BamHI/HindIII* pattern of pSA30—only the *Klebsiella* subfragments *a* and *b* hybridized. (2) Vector pBR322 was used as a hybridization probe for restriction fragments of pRJ676, and only DNA fragments containing parts of pBR322 hybridized. (3) The *EcoRI* fragment of pSA30 was used to probe some of the individual subclones of pRJ676, verifying the approximate region of homology described here. (4) The 4.85×10^6 MW *R. japonicum* DNA was found to hybridize to plasmid pGR151, which contains a cloned *Klebsiella* DNA fragment coding for the amino-terminal part of the *nifH* gene product (G. E. Riedel, unpublished results). This supports the finding that pRJ676 is homologous to *nifH* of *K. pneumoniae*.

Thus, Fig. 1 shows the approximate region of homology between the *K. pneumoniae* and *R. japonicum nif* DNAs. Ruvkun and Ausubel⁵ reported the successful cloning of part of the *nif* genes from *R. meliloti*. Their results agree well with ours, and it is intriguing that only two genes out of the 17 *Klebsiella nif* genes share apparent DNA sequence homology. It will be interesting to determine whether the cloned DNA regions of pRJ676 adjacent to the *nif*-homology region code for proteins of the symbiotic nitrogen-fixing apparatus. The availability of cloned *R. japonicum nif* DNA in *E. coli* makes it possible to alter this region genetically, and preliminary experiments have shown that transposable DNA elements¹³ can be introduced easily into the cloned *R. japonicum nif* DNA (our unpublished results).

This work was sponsored by a grant from the Deutsche Forschungsgemeinschaft. I thank Dr A. Böck for support, Drs F.

Ausubel, F. Cannon, G. Riedel, G. Ruvkun and U. Rüdter for bacterial strains and helpful suggestions, and P. Frischeisen, M. Fuhrmann and K. Kaluza for technical assistance.

Received 21 November 1980; accepted 6 March 1981.

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Synthesis and characterization of (2'-5')ppp3'dA(p3'dA)_n, an analogue of (2'-5')pppA(pA)_n

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The secretion of interferon after viral infection enhances antiviral resistance in other cells characterized by increased activity of certain enzymes, a protein kinase, an endoribonuclease and a (2'-5') (A)_n synthetase^{1–6}. The biochemical function of the synthetase is to convert ATP into oligonucleotides called generically (2'-5')pppA(pA)_n, which are characterized by the 2',5'-phosphodiester bond (see Table 1 legend for abbreviations used). The function of these (2'-5') oligonucleotides seems to be the activation of the latent endoribonuclease whose consequence is to inhibit protein synthesis. As part of our study of the development of the antiviral/antitumour state, we have set out to synthesize analogues of (2'-5')pppA(pA)_n, in the expectation that these analogues would also activate a latent endoribonuclease, yet be metabolically more stable. We now describe the enzymatic conversion of 3'dATP (cordycepin 5'-triphosphate) to the 5'-triphosphate trimer, (2'-5')ppp3'dA(p3'dA)₃, the effectiveness of the latter in the inhibition of translation and its resistance to hydrolysis by 2',5'-phosphodiesterase⁷. The enzymatic synthesis of the 2',5'-phosphodiester bond with 3'dATP by (2'-5') (A)_n synthetase is an extension of our earlier report on the formation of a 2',5'-phosphodiester bond following the incorporation of 3'dAMP from [G-³H]cordycepin into the internucleotide linkage of RNA of H.Ep. 1 cells⁸.

The per cent conversions of ATP and 3'dATP to (2'-5') oligonucleotides following 5- and 17-h incubations are shown in Table 1. The radioactive oligonucleotides synthesized following incubations with either ATP or 3'dATP eluted from the poly(rI)-poly(rC)-agarose columns were diluted to 50 mM KCl and analysed on DEAE-cellulose in a 7 M urea-NaCl gradient with authentic (2'-5')pppA(pA)₂ (P-L Biochemicals). Six radioactive compounds were detected following incubations with [G-³H]3'dATP (Fig. 1a). One, with a charge of -6, was displaced together with authentic marker (2'-5')pppA(pA)₂ (peak tube at 36 ml, Fig. 1a). The ³H-nucleotide, with a charge

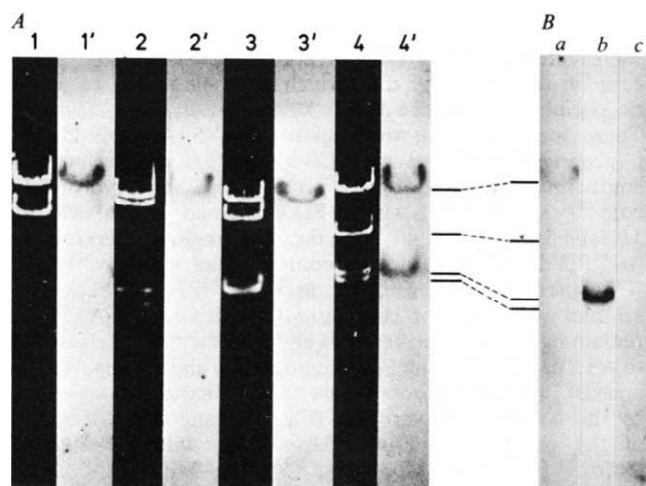


Fig. 2 Hybridization of DNA fragments of pRJ676 with ³²P-labelled *K. pneumoniae nif* DNA. A, Restriction fragments were generated by digestion with the enzymes *HindIII* (1), *BamHI* (2), *HindIII/BamHI* (3) and *EcoRI* (4). They were then separated on 0.8% agarose gels and stained with ethidium bromide. Duplicates were transferred on to nitrocellulose filters and hybridized with the nick-translated *EcoRI* fragment of pSA30. The white strips (1', 2', 3' and 4') show the respective autoradiograms. B, *EcoRI* fragments of pRJ676 were hybridized in three separate experiments with nick-translated subfragments *a*, *b* and *c* of pSA30 (compare with Fig. 1). Only the respective autoradiogram strips are shown.

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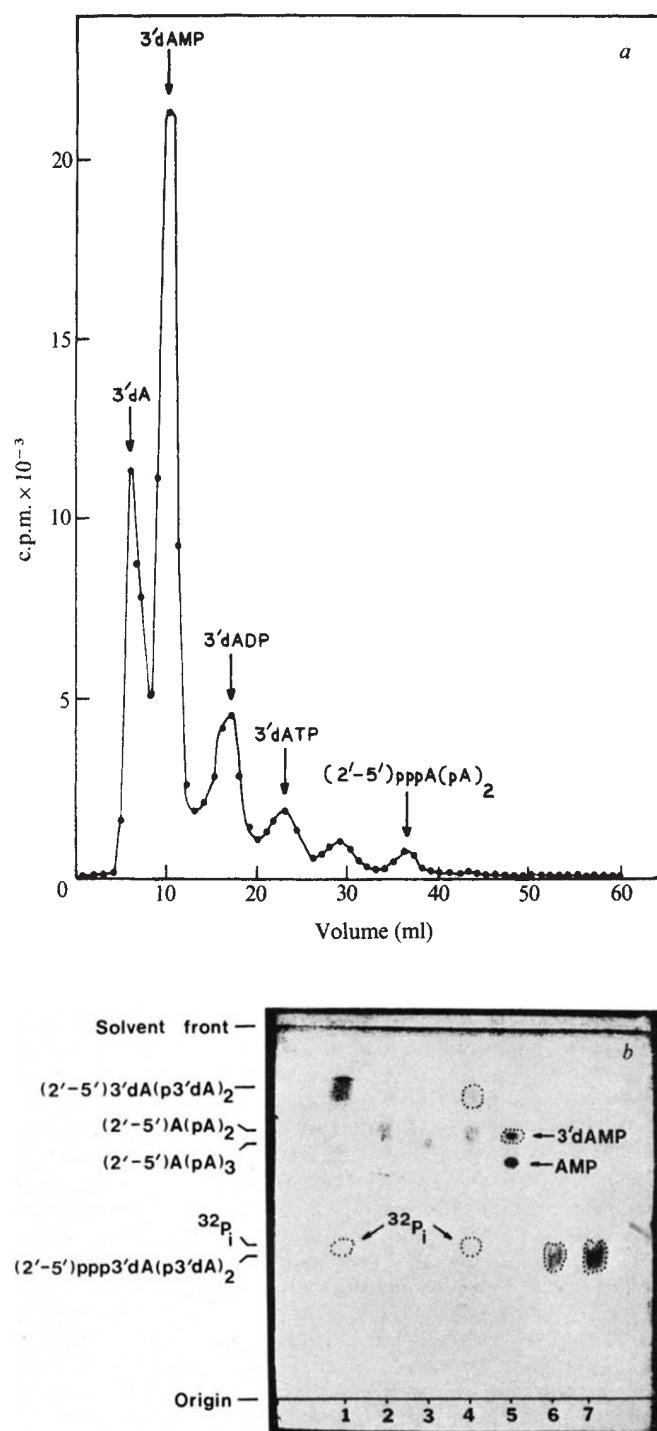


Fig. 1 *a*, Chromatography of the analogue, $^3\text{H}-(2'-5')\text{ppp}3'\text{dA}(\text{p}3'\text{dA})_2$, from incubations with $[\text{G}-^3\text{H}]3'\text{dATP}$ on DEAE-cellulose. Peak fractions of material washed from poly(rI)-poly(rC)-agarose columns from a 17-h incubation with $[\text{G}-^3\text{H}]3'\text{dATP}$ were applied to a DEAE-cellulose column (Whatman DE52, 0.5×17 cm). The nucleotides were displaced with a 50–150 mM linear gradient of NaCl (40 ml/40 ml) and 50 mM Tris-HCl, pH 8.0, in 7 M urea; 1-ml fractions were collected; flow rate 4 ml h^{-1} . Cordycepin ($3'\text{dA}$), $3'\text{dAMP}$, $3'\text{dADP}$, $3'\text{dATP}$ and authentic $(2'-5')\text{pppA}(\text{pA})_2$ were included as markers as indicated by arrows and were monitored on a recording spectrophotometer (LKB 2138 Uvicord S). The elution profile represents a 0.10-ml aliquot of each 1-ml fraction assayed for radioactivity. A similar elution profile was obtained from a 17-h incubation with $[\alpha-^{32}\text{P}]3'\text{dATP}$ (data not shown). In the other incubations with $[\alpha-^{32}\text{P}]3'\text{dATP}$ and $[\text{G}-^3\text{H}]3'\text{dATP}$, the radioactive material eluting from the poly(rI)-poly(rC)-agarose columns was chromatographed on DEAE-cellulose columns (0.6×2.0 cm) and washed with 42 ml of buffer B: 0.09 M KCl, 20 mM HEPES, pH 7.5. The oligonucleotides were displaced with 5 ml of buffer B supplemented with 0.35 M KCl. The radioactive nucleotides in the 0.35 M KCl were further fractionated by the 7 M urea-NaCl gradient-DEAE-cellulose chromatography method described above. *b*, Photograph taken under UV light of cellulose TLC (Eastman no. 13254) developed in solvent A. Dark areas indicate authentic (chemically synthesized) nucleotides detected by UV light; dotted circles indicate areas of ^{32}P . Following photography, lanes 1–7 from the origin to the solvent front were cut into 1-cm² pieces, eluted with water and assayed for radioactivity in 5 ml aqueous counting scintillant (Amersham). Lane 1: authentic unlabelled core $(2'-5')3'\text{dA}(\text{p}3'\text{dA})_2$ (R_F 0.81) detected by UV light and $^{32}\text{P}_i$ (standard, R_F 0.41) indicated by dotted circle. Lanes 2, 3: authentic unlabelled core $(2'-5')\text{A}(\text{pA})_2$ (R_F 0.71) and authentic unlabelled core $(2'-5')\text{A}(\text{pA})_3$ (R_F 0.68), respectively, detected by UV light. Lane 4: bacterial alkaline phosphatase digest of $^{32}\text{P}-(2'-5')\text{ppp}3'\text{dA}(\text{p}3'\text{dA})_2$; core $^{32}\text{P}-(2'-5')3'\text{dA}(\text{p}3'\text{dA})_2$ (R_F 0.81, 2,397 d.p.m.) and $^{32}\text{P}_i$ (R_F 0.40, 2,829 d.p.m.) indicated by dotted circles (digestion did not go to completion; when digestion of similar preparations did go to completion, 34% of the ^{32}P was isolated as $^{32}\text{P}_i$ by XAD-4 chromatography, see above); authentic unlabelled core $(2'-5')3'\text{dA}(\text{p}3'\text{dA})_2$ (R_F 0.81), authentic unlabelled core $(2'-5')\text{A}(\text{pA})_2$ (R_F 0.71) and authentic unlabelled core $(2'-5')\text{A}(\text{pA})_3$ (R_F 0.68) detected by UV light (no radioactivity was detected in the core $(2'-5')\text{A}(\text{pA})_2$ or core $(2'-5')\text{A}(\text{pA})_3$ regions). Lane 5: snake venom phosphodiesterase digest of core $^{32}\text{P}-(2'-5')3'\text{dA}(\text{p}3'\text{dA})_2$; $^{32}\text{P}-3'\text{dAMP}$ (R_F 0.69, 1,228 d.p.m.) indicated by dotted circles; authentic $3'\text{dAMP}$ (R_F 0.69) and AMP (R_F 0.62) detected by UV light (no radioactivity was detected in the AMP region). Lane 6: $^{32}\text{P}-(2'-5')\text{ppp}3'\text{dA}(\text{p}3'\text{dA})_2$ (R_F 0.36, 1,729 d.p.m.) indicated by dotted circle and authentic $(2'-5')\text{pppA}(\text{pA})_2$ (R_F 0.36) detected by UV light. Lane 7: T2 RNase digest of $^{32}\text{P}-(2'-5')\text{ppp}3'\text{dA}(\text{p}3'\text{dA})_2$; the $^{32}\text{P}-(2'-5')\text{ppp}3'\text{dA}(\text{p}3'\text{dA})_2$ (R_F 0.36, 1,722 d.p.m.) indicated by dotted circle co-chromatographed with authentic marker $(2'-5')\text{pppA}(\text{pA})_2$ (R_F 0.36 detected by UV light). In control experiments, 0.5% core $^{32}\text{P}-(2'-5')\text{A}(\text{pA})_3$ could be detected when mixed with 99.5% core $^{32}\text{P}-(2'-5')3'\text{dA}(\text{p}3'\text{dA})_2$ and chromatographed (Whatman 3MM, solvent A).

of -6, has been identified as the triphosphate trimer analogue of $3'\text{dATP}$, that is, $(2'-5')\text{ppp}3'\text{dA}(\text{p}3'\text{dA})_2$.

Several experiments were done to characterize the structure of the putative $(2'-5')\text{ppp}3'\text{dA}(\text{p}3'\text{dA})_2$ and to establish its biological properties. Treatment of the putative $^{32}\text{P}-(2'-5')\text{ppp}3'\text{dA}(\text{p}3'\text{dA})_2$ with bacterial alkaline phosphatase (BAP) followed by XAD-4 chromatography⁹ resulted in the isolation of 34% of the ^{32}P as $^{32}\text{P}_i$ (theoretical, 33.3%). Additional proof for the structure of $(2'-5')\text{ppp}3'\text{dA}(\text{p}3'\text{dA})_2$ was obtained by treatment of the adenylate analogue with BAP, snake venom phosphodiesterase I (SVPD I) and T2 RNase followed by TLC and measurement of radioactive compounds (Fig. 1b). The nucleotide synthesized from $[\alpha-^{32}\text{P}]3'\text{dATP}$ and purified by DEAE-cellulose chromatography (Fig. 1a, charge -6) showed only one radioactive spot (Fig. 1b, lane 6). BAP hydrolysis¹⁰ of

the putative $[\alpha-^{32}\text{P}](2'-5')\text{ppp}3'\text{dA}(\text{p}3'\text{dA})_2$ produced $^{32}\text{P}_i$ and core $^{32}\text{P}-(2'-5')3'\text{dA}(\text{p}3'\text{dA})_2$ which had the same R_F as authentic, chemically pure core $3'\text{dA}(\text{p}3'\text{dA})_2$ (ref. 11) (Fig. 1b, lane 4). There was no ^{32}P in the region of $(2'-5')\text{A}(\text{pA})_3$ (lane 4), indicating that putative $^{32}\text{P}-(2'-5')\text{ppp}3'\text{dA}(\text{p}3'\text{dA})_2$ was not contaminated with $(2'-5')\text{ppA}(\text{pA})_3$ (charge -6). Hydrolysis of core $^{32}\text{P}-3'\text{dA}(\text{p}3'\text{dA})_2$ with SVPD I¹⁰ formed $^{32}\text{P}-3'\text{dAMP}$ (Fig. 1b, lane 5); there was no ^{32}P in the AMP region. Therefore, the $[\alpha-^{32}\text{P}]3'\text{dATP}$ could not be contaminated with $[\alpha-^{32}\text{P}]3'\text{dATP}$. (Of eight commercial preparations of $[\alpha-^{32}\text{P}]3'\text{dATP}$, only two samples were free of contamination with $[\alpha-^{32}\text{P}]3'\text{dATP}$. The remaining six preparations showed ^{32}P in the regions equivalent to ATP or AMP by paper chromatography and TLC (solvents A and B).) Further proof of the 2', 5' linkage was obtained by the absence of hydrolysis (Fig. 1b, lane 7) on treatment of the putative $(2'-5')\text{ppp}3'\text{dA}(\text{p}3'\text{dA})_2$ with T2 RNase¹⁰. Core $^{32}\text{P}-(2'-5')3'\text{dA}(\text{p}3'\text{dA})_2$, $^{32}\text{P}-3'\text{dAMP}$ and $^{32}\text{P}-(2'-5')\text{ppp}3'\text{dA}(\text{p}3'\text{dA})_2$ (Fig. 1b, lanes 4–6, respectively) following elution and rechromatography on cellulose TLC plates in solvent B showed only one radioactive spot each which co-chromatographed with their respective standards.

The inhibition of translation by the analogue $(2'-5')\text{ppp}3'\text{dA}(\text{p}3'\text{dA})_2$ was compared with the inhibition by tetramer adenylate $(2'-5')\text{pppA}(\text{pA})_3$ (Fig. 2a) and the trimer adenylate $(2'-5')\text{pppA}(\text{pA})_2$ (Fig. 2b). The inhibition of translation by 40 nM $(2'-5')\text{pppA}(\text{pA})_3$ was 52% and decreased to control values at 0.004 nM; that by 22 nM $(2'-5')\text{ppp}3'\text{dA}(\text{p}3'\text{dA})_2$ was 68% and decreased to control

Table 1 Enzymatic synthesis of (2'-5')ppp3'dA(p3'dA)_n and (2'-5')pppA(pA)_n

Substrate	Final concentration (mM)	Incubation time (h)	Conversion to (2'-5')oligonucleotide (%)
[8- ³ H]ATP	2	5	21.9
[8- ³ H]ATP	2	17	19.0
[α- ³² P]3'dATP	2	5	0.3
[α- ³² P]3'dATP	2	17	3.0
[G- ³ H]3'dATP	1.3	17	3.7

Poly(rI)·poly(rC)-agarose columns (0.5 × 1.5 cm) bound with (2'-5')(A)_n synthetase were prepared as described previously¹⁹, except that reticulocyte lysate (1.2 ml) was used in place of HeLa cell extract and the column wash buffer consisted of 100 mM KCl, 2 mM Mg(OAc)₂, 2 mM dithiothreitol, 10% glycerol and 20 mM HEPES, pH 7.5 (buffer A). The final concentration of Mg(OAc)₂ was 8 mM in all incubations except for the [G-³H]3'dATP experiment where the concentration was 2.3 mM. Columns were incubated at 30 °C for the time periods indicated with [8-³H]ATP (10 μCi, 22 Ci mmol⁻¹), [α-³²P]3'dATP (25 μCi, 640 or 3,000 Ci mmol⁻¹, Amersham, NEN) or [G-³H]3'dATP (10 μCi, 640 mCi mmol⁻¹). Ninety-five per cent of the radioactivity was eluted with 2 ml of buffer A. Cordycepin (3'-deoxyadenosine) was isolated and purified from *Cordyceps militaris* (m.p. 228–229 °C, literature 231 °C)²⁰. Commercially available 3'dATP of the highest purity was purchased (from Sigma) or synthesized in this laboratory²¹. [G-³H]3'dATP was synthesized in this laboratory, oxidized with periodate to remove contaminating ATP or other ribonucleotides and repurified by descending paper chromatography (Whatman 3MM in solvents A and B; solvent A: isobutyric acid/concentrated ammonia/water, 66:1:33, v/v/v; solvent B: 1-propanol/concentrated ammonia/water, 60:30:10, v/v/v). In solvents A and B, one UV light absorbing region or one radioactive region was detected for 3'dATP, [G-³H]3'dATP or [α-³²P]3'dATP. The ratio of tritium in the adenine:3-deoxy-ribose of the [G-³H]cordycepin was 9:1 (ref. 21). Product formation was determined by the amount of radioactivity displaced from the DEAE-cellulose column with 0.35 M KCl divided by the total radioactivity recovered, except for the [G-³H]3'dATP experiment. Per cent conversion for the [G-³H]3'dATP experiment was determined from the elution profile shown in Fig. 1a as the percentage of radioactivity eluting after 3'dATP because chromatography of this material on DEAE-cellulose and elution with 0.35 M KCl was not performed before the DEAE-cellulose chromatography NaCl gradient elution method shown in Fig. 1a. The amount of ³²P displaced with 0.35 M KCl when 25 μCi of [α-³²P]3'dATP was added to DEAE-cellulose columns was 0.15%, which was subtracted from the ³²P in the 0.35 M KCl fraction. Abbreviations used are: A, adenosine; 3'dA, 3'-deoxyadenosine (cordycepin); pA, 5'AMP; p3'dA, 3'-deoxy-5'-AMP; 3'dATP, 3'-deoxyATP; (2'-5')pppA(pA)₂, (2'-5')pppA(pA)₂, or (2'-5')ppp3'dA-(p3'dA)₂, the 2',5'-linked trimers or tetramer of either AMP or 3'dAMP with a triphosphate at the 5' end; core, (2'-5')A(pA)_n or (2'-5')3'dA(p3'dA)_n.

values at 0.04 nM (Fig. 2a). The trimer analogue (2'-5')ppp3'dA-(p3'dA)₂ is about four times more potent an inhibitor of translation than is (2'-5')pppA(pA)₂ (Fig. 2b). The inhibition of translation by (2'-5')ppp3'dA(p3'dA)₂ ± periodate did not change, although there was a small change in the inhibition of translation by periodate-treated (2'-5')pppA(pA)₂ (Fig. 2b). These data indicate that there is no ribosyl moiety on the 3' terminus of (2'-5')ppp3'dA(p3'dA)₂. In comparing the inhibition of translation by (2'-5')ppp3'dA(p3'dA)₂ with that of (2'-5')pppA(pA)₂ (Fig. 2b), it must be kept in mind that Williams *et al.*¹² reported that in the lysed rabbit reticulocyte system (2'-5')pppA(pA)₂ is not a good inhibitor of translation. The translation experiments show inhibition of translation by 0.4 nM analogue (2'-5')ppp3'dA(p3'dA)₂, but no inhibition by 0.004 nM (2'-5')pppA(pA)₃; thus, any possible contamination of [α-³²P]3'dATP with [α-³²P]ATP must be <1%.

The stability of (2'-5')ppp3'dA(p3'dA)_n in HeLa cell extracts was compared with that of (2'-5')pppA(pA)_n. There was no hydrolysis of the analogue (2'-5')ppp3'dA(p3'dA)_n (12 μM) by HeLa cell extracts¹³ following a 45-min incubation; however, the t_{1/2} of (2'-5')pppA(pA)_n (5 μM) is about 2 min (Fig. 3). With 500 μM (2'-5')pppA(pA)_n, there was a 50% hydrolysis in 45 min as determined by DEAE-cellulose chromatography; with 500 μM authentic core analogue (2'-5')3'dA(p3'dA)₂ there was no hydrolysis as determined by cellulose TLC (solvents A and B). Neither cordycepin nor 3'dAMP was detected. The lack of hydrolysis of the analogue may be similar to the lack of hydrolysis of (3'-5')2'-deoxyribonucleotides by the 2', 5'-phosphodiesterase¹⁴.

Thus, the data presented show that (1) the analogue of (2'-5')pppA(pA)_n can be synthesized enzymatically from 3'dATP by the (2'-5')(A)_n synthetase from lysed rabbit reticulocytes, (2) the 3'-hydroxyl on the ribose moiety of ATP

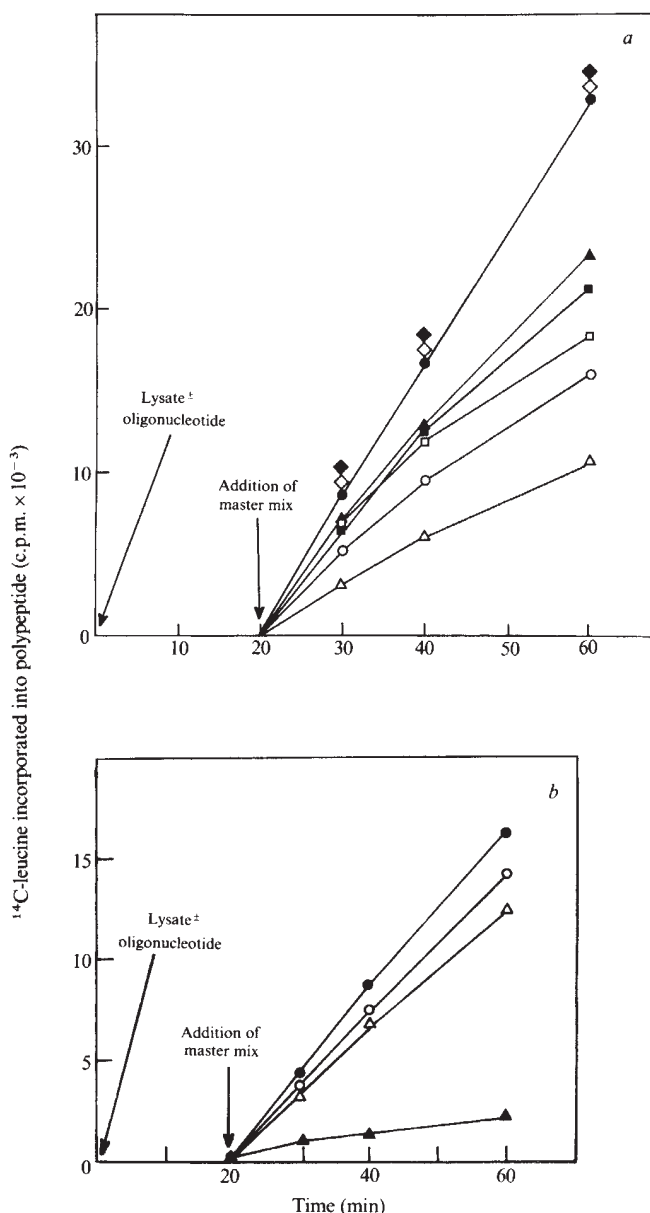


Fig. 2 a, Inhibition of translation by (2'-5')pppA(pA)₃ (tetramer adenylylate) at 40 nM (O), 0.4 nM (□), 0.04 nM (■), and 0.004 nM (◆) were compared with trimer 3'-deoxyadenylate at 22 nM (Δ), 0.4 nM (▲), 0.004 nM (◇) and control (●). Concentrations are expressed in terms of AMP and 3'dAMP equivalents. (2'-5')pppA(pA)₃ was synthesized and isolated as described elsewhere²³. (2'-5')ppp3'dA(p3'dA)₂ was obtained by DEAE-cellulose chromatography (Fig. 1a, marker (2'-5')pppA(pA)₂ was omitted in this purification). The fractions corresponding to putative ³²P-(2'-5')ppp3'dA(p3'dA)₂ from the NaCl gradient elution method were pooled, desalted by gel filtration (Sephadex G-10, 0.9 × 87 cm), and concentrated *in vacuo*. Translation was assayed by the incorporation of L-[U-¹⁴C]leucine (0.60 μCi, 335 mCi mmol⁻¹) into trichloroacetic acid-insoluble polypeptides. The data were obtained by using 5-μl aliquots of the assay volume (30 μl) processed as described previously²⁴. Oligonucleotide samples in 3.0 μl were preincubated in the rabbit reticulocyte lysate (15 μl, Clinical Convenience Inc.) for 20 min before the start of translation²⁴. Master mix components and their final concentrations in the lysate were as follows: ATP, 1.0 mM; GTP, 0.2 mM; phosphocreatine, 15 mM; creatine phosphokinase, 1.3 units per 30 μl; KCl, 85 mM; haemin, 25 μM. b, Inhibition of translation by (2'-5')pppA(pA)₂ (trimer adenylylate) and (2'-5')ppp3'dA(p3'dA)₂ (analogue trimer 3'-deoxyadenylate). Inhibition of translation with (2'-5')pppA(pA)₂ (100 nM) (Δ) and 100 nM plus periodate (●) were compared with that of (2'-5')ppp3'dA(p3'dA)₂ (100 nM) or 100 nM plus periodate (▲) and control (●). (2'-5')ppp3'dA(p3'dA)₂ was isolated as described above. Translation assays were performed as described above except that 0.25 μCi of L-[U-¹⁴C]leucine (355 mCi mmol⁻¹) was used and the assay volume was 50 μl. Oligonucleotide samples (5 pmol) were treated with 1.25 nmol of sodium metaperiodate²² in a final volume of 10 μl for 20 min at 25 °C in the dark. Periodate-treated oligonucleotides were tested in translation assays as described above. Periodate in samples containing no oligonucleotide did not inhibit translation.

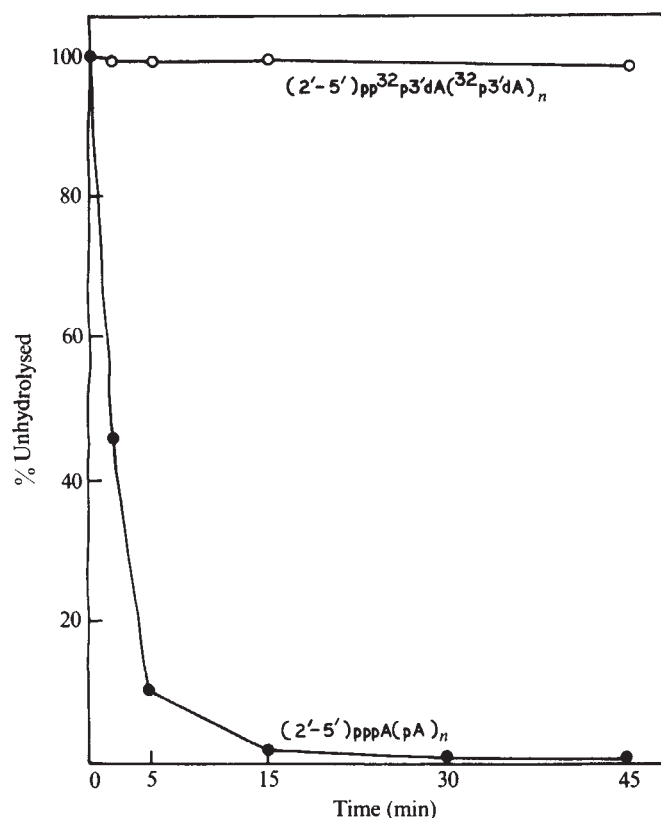


Fig. 3 Stability of (2'-5')pppA(pA)_n and (2'-5')ppp3'dA(p3'dA)_n in HeLa cell extracts. ³H-(2'-5')pppA(pA)_n and ³²P-(2'-5')ppp3'dA(p3'dA)_n were synthesized as described in Table 1 legend. The oligonucleotides were obtained from the 0.35 M KCl eluate (DEAE-cellulose chromatography), desalted (Sephadex G-10) and concentrated *in vacuo*. The methods used to determine degradation of oligonucleotides were a modification of that of Minks *et al.*¹³. Incubation mixtures (0.125 ml) contained 0.6 parts of HeLa cell extract prepared as described previously²⁵, 2.5 mM Mg(OAc)₂, either ³²P-(2'-5')ppp3'dA(p3'dA)_n (12 μM) or ³H-(2'-5')pppA(pA)_n (5 μM), 120 mM KCl, 20 mM HEPES pH 7.4, and 1 mM dithiothreitol. Incubations were performed at 30°C. At the times indicated, 25-μl samples were withdrawn, heated for 3 min at 95°C, and the amount of undegraded oligonucleotide was determined by DEAE-cellulose chromatography as described previously¹³. Degradation of oligonucleotides was monitored as a decrease of radioactive material eluting with 0.35 M KCl compared with the zero time point (100% undegraded).

is important but not essential for the synthesis of (2'-5')pppA(pA)_n, (3) (2'-5')ppp3'dA(p3'dA)₂ is a more potent inhibitor of translation than (2'-5')pppA(pA)₂, (4) (2'-5')ppp3'dA(p3'dA)₂ and (2'-5')pppA(pA)₃ are approximately equal in their abilities to inhibit translation, and (5) enzymatically synthesized analogue (2'-5')ppp3'dA(p3'dA)_n and chemically synthesized core analogue (2'-5')3'dA-(p3'dA)₂ are not hydrolysed by the 2', 5'-phosphodiesterase in HeLa cell extracts. 3'dATP is also converted to (2'-5')ppp3'dA-(p3'dA)_n by extracts of human fibroblast interferon-treated L cells (manuscript in preparation). Enzymatically and chemically synthesized core (2'-5')3'dA(p3'dA)₂ inhibits transformation of Epstein-Barr virus (EBV)-infected lymphocytes in studies with human umbilical cord lymphocytes infected with EBV (manuscript in preparation).

The replacement of ATP with 3'dATP as a substrate for (2'-5')(A)_n synthetase has been reported from three other laboratories. Lengyel and co-workers showed that (2'-5')(A)_n synthetase from interferon-treated EAT cells can link adenylate moieties to the 2'-hydroxyl of 3'dATP¹⁵. Justesen *et al.* reported that 3'dATP is a chain terminator with respect to the addition of one 3'-deoxyadenylate moiety to either dimer or trimer (2'-5')pppA(pA)_n following 3-h incubations with rabbit reticulocyte (2'-5')(A)_n synthetase¹⁶. Minks *et al.* reported that

3'dATP inhibits the HeLa cell (2'-5')(A)_n synthetase in the presence of ATP with respect to formation of (2'-5')pppA(pA)_n (ref. 17). Baglioni and co-workers¹⁸ have also reported that the ATP analogue, 1-N⁶-ethenoATP, is converted to (2'-5')oligonucleotides which activate HeLa cell endonuclease and degrade vesicular stomatitis virus RNA. As (2'-5')oligonucleotides have been shown to inhibit mitogen-stimulated DNA synthesis in lymphocytes, as does interferon¹⁹, it is tempting to speculate that analogues of (2'-5')pppA(pA)_n may potentiate, extend or regulate these activities and could possibly be used as chemotherapeutic agents which might replace interferon.

The structure of the compound eluting at 29 ml on the DEAE cellulose column (Fig. 1a) is now under investigation.

We thank Nancy Lee Reichenbach for technical assistance in the isolation of cordycepin and the chemical synthesis of 3'dAMP and 3'dATP, Dr Wolfgang Pfeleiderer for the supply of authentic chemically synthesized (2'-5')3'dA(p3'dA)₂, and Dr D. S. Rabson for human fibroblast interferon. This research was supported in part by NIH and NSF grants.

Received 10 April 1980; accepted 5 March 1981.

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Erratum

In the letter 'Scale of body pattern adjusts to available cell number in amphibian embryos' by Jonathan Cooke, *Nature* **290**, 775-778 (1981), brackets in Table 1 were meant to refer to duplications of control or experimental embryos within each matched set. As these brackets are partially misaligned, they should be ignored and the data read by the use of a horizontal rule to lead the eye across the page in the absence of gaps between the data of each set.

Nature Indexes

Author indexes to *Nature* are published bimonthly, in the third week following the end of each volume. A complete subject and author index is published at the end of each year. Author indexes published this year are those for volumes 289 (26 March) and 290 (21 May).

MATTERS ARISING

Polygon patterns on Europa

PIERI¹, in his study of the lineament and polygon patterns on Europa, has discussed the problem of the development of crack networks in cooling basalt flows and similar planar, contracting systems. He observed that although an ideal hexagonal pattern maximizes the reduction of strain energy per unit surface area and is therefore the most efficient pattern given an isotropic stress field, nevertheless, even well developed columnar basalts tend to show many pentagons along with the expected hexagons.

The observations by Beard² on basalt flows the data used by Pieri certainly show a large proportion of pentagons, ranging from 50% at Mount Rodeix to 35% at the Giants Causeway. Beard also recorded the mean number of sides of the basalt polygons and obtained values ranging from 5.23 at Mount Rodeix to 5.66 at the Giants Causeway. In an ideal system, with only 3-rayed crack vertices the mean side number should be six. A simple Monte Carlo model of a contracting cooling basalt can explain the significant occurrence of non-hexagons and also the reason why Beard's side counts were less than the ideal six.

In natural conditions, in an essentially unbounded basalt flow, one would expect the crack pattern to begin forming in a random manner; conditions can never be ideal enough for the entire pattern to develop in one cracking operation. Thus it can be expected to grow from randomly distributed stress centres³. Using a simple random number placement method and an effectively infinite field crack pattern, models can be produced which show many of the features observed by Beard. The model networks have a significant number of pentagons and heptagons and with just the random condition operating always have a mean polygon side number of six. However, a small number of extremely short sides are always produced and if these are eliminated the mean side value drops to ~5.6–5.7.

This suggests that Beard was unable to detect the very shortest sides and that this accounts for his mean side values in the 5.6 region. If this is so then a flow like the Giants Causeway is very close to an ideal flow; the ideal flow has a mean side number of six but as nature favours random processes we should expect a reasonable proportion of pentagons and heptagons. Pieri stated that the more isotropic and uniform the stress, the more likely it will be that hexagons form: this is looking to an ideal-ideal situation; in a real-ideal situation (a possible geological happening) it is likely that the mean side

number will approach six but we should still expect non-hexagons to appear in the network.

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1. Pieri, D. C. *Nature* **289**, 17–21 (1981).
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PIERI replies—It is clear from Smalley's comment above and from previous work¹ that polygonal fracture patterns forming in real materials in even the most ideal of isotropic stress regimes are subject to compositional anisotropies at many scales and a fracture network cannot form all at once. Thus, the ideal perfectly-formed pure-hexagonal pattern will never form in nature and will have particular difficulty in the context of global planetary fracture patterns². I did not mean to imply that the end result of optimized real conditions would be pure hexagonal patterns, but rather that hexagons would be more numerous in situations where conditions were more uniform. Clearly, pentagons and heptagons will still be present, and I thank Smalley for clarifying this point.

The research described in this note was carried out by the Jet Propulsion Laboratory, California Institute of Technology, under contract with NASA.

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1. Smalley, I. J. *Geol. Mag.* **10**, 110–114 (1966).
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Low-angle X-ray scattering of chromatin

LANGMORE AND SCHUTT¹ have recently reported low-angle X-ray scattering experiments on chicken erythrocytes in which they find a peak at ~400 Å in the plot of s^2I against s . They conclude that this peak is related to the side-by-side packing of chromosome fibres. I wish to show here that this peak may have a different interpretation.

Langmore and Schutt¹ multiply the recorded intensity I by s^2 to correct for the random orientation of the fibres with respect to the X-ray beam, where $s = 2 \sin(\theta/2)/\lambda$. However, as shown quite generally by Porod² and discussed in detail by Mittelbach³, information on the cross-section properties of rod-like parti-

cles is best obtained from the study of the dependence of I_s (instead of I_s^2) on s .

At very low angles, a system of rod-like particles obeys the approximate Guinier equation:

$$(I_s) = K \exp[-2(\pi s R_G)^2] \quad (1)$$

where K is a constant and R_G the radius of gyration of the cross-section. For a cylindrical particle of uniform density and radius R , $R_G = 0.707 R$. If I_s^2 is plotted instead of I_s , a maximum in the curve will appear when $d(I_s^2)/ds = 0$. Introducing this condition in equation (1), it is easily shown that a maximum in the plot of I_s^2 against s should appear for

$$s = 1/2\pi R_G \quad (2)$$

Therefore, the maximum found by Langmore and Schutt¹ in the plots of I_s^2 against s for $s = 1/400 \text{ Å}^{-1}$ is consistent with the presence of cylindrical objects with a cross-sectional radius of gyration $R_G = 64 \text{ Å}$, as calculated from equation (2). This value would correspond to cylindrical particles of 180-Å diameter if their electron density were uniform. Thus I think that the peak observed by Langmore and Schutt¹ is due to intrinsic features of the chromatin fibres and does not demonstrate any regular side-by-side packing of chromosome fibres.

I suggest that the determination of the position of maxima and the use of equation (2) may be a useful alternative to Guinier plots in determining the value of R_G . A similar procedure could be used for globular particles, but in that case the maxima in the plot of I_s against s would be used.

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LANGMORE AND SCHUTT REPLY—Although it is necessary that widely spaced homogeneous cylindrical fibres of diameter D give rise to a maximum in s^2I at $\sim s = (2.2 D \text{ Å})^{-1}$, a peak in the s^2I curve is not sufficient to prove the existence of widely spaced homogeneous cylindrical particles. The analysis that Subirana suggested is only relevant to the determination of the radius of gyration of particles provided that these particles are rod-like and widely separated. Proper Guinier analysis¹ tests the validity of these

necessary conditions by comparing the shape of the experimental scattering curve with that predicted for widely separated rod-like particles. Such analysis of the scattering from our molecules shows that the Guinier conditions are not met, because the experimental plot of $\log sI$ against s^2 is not linear, due to the fact that the chromosome fibres we have studied are not widely separated (*vide infra*).

In our letter we outlined three basic arguments to prove that the 400 Å band is due to the packing of fibres and therefore not the result of scattering from widely separated rod-like molecules. These arguments were that: (1) the 400-Å peak is found in the I versus s plots of living chicken erythrocytes after subtraction of the non-nuclear background (represented by the scattering from living rabbit erythrocytes); (2) the 400-Å band in the s^2I vs s plots can be directly related to the 300-Å centre-to-centre spacing of fibres often seen in thin sections of a wide range of cells, including chicken erythrocytes²; and (3) the 400-Å peak in the s^2I curves of the scattering from intact nuclei is lost when the thick fibres are intentionally disaggregated into soluble thick fibres (as assayed by our electron microscopy) by elimination of divalent cations from the buffer. Each of these arguments disproves that scattering from isolated thick fibres has given rise to the 400-Å features in our patterns.

If, as we suggest, erythrocyte chromosome fibres are homogeneous cylinders of 370–400 Å diameter, a dilute dispersion of fibres would give rise to a maximum in s^2I at ~ 850 Å. To see a peak at these very small angles would require better first-order resolution in our X-ray camera.

Thus we feel that our experiments have demonstrated that the 400-Å feature is due exclusively to the side-by-side packing of chromosome fibres. The absence of a 400-Å feature in disaggregated fibres is inconsistent with the hypothesis put forth by Subirana. Furthermore the analytical technique he has proposed is not generally valid because it cannot test whether the necessary conditions for a Guinier analysis are met.

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Stability of loess in light of the inactive particle theory

A RECENT article by Smalley *et al.*¹ has suggested an interesting theory pertaining to loess stability. The inactive particle theory of soil sensitivity attempts to explain the sensitivity of quickclays on the basis that these soils possess only small amounts of clay minerals and as a result, develop no long-range bonds. Therefore once disturbed, they do not retain the adequate shear strength needed to remain stable^{2,3}.

In the classical Terzaghi sense, loess soils, which are composed predominantly of silt particles, would probably not be considered sensitive soils, in that the ratio of undisturbed to remolded strength, at constant moisture content, is usually around 3, depending on clay content, and therefore would generally fall into the category of medium sensitivity. If, however, sensitivity is taken as the ratio of undisturbed to saturated strength (in unconfined compression) as indirectly suggested by Feda⁴, then some loess soils would have to be considered quick.

Loess soils, particularly bluff-line deposits, typically lose shear strength as a result of moisture saturation; in which case landslide potential becomes an extreme hazard. Loess in this state may be susceptible to "spontaneous liquefaction"⁵ which could help explain the extent of landslides during the 1920 earthquake in the Kansu Province of China.

In recent investigations throughout the midwestern US, *in-situ* stability of loess has been related to liquidity index, that is when the *in-situ* moisture content reaches the liquid limit, usually on saturation, stability is all but lost and can be readily identified by isolated flow in boreholes. Because liquid limit is related to clay content, and saturation moisture is a function of density, this instability only occurs in special circumstances, typically low density and low clay content. As both calcareous and leached examples of this condition have been noted, the leaching theory of Rosenquist does not seem applicable in that carbonate leaching need not be complete, however, the cementation bond may be weakened. Scanning electron microscopy (SEM) qualitatively reveals that only a small portion of the clay fraction ($<2 \mu\text{m}$) of loess is composed of clay minerals, with the remainder being comprised of clay sized quartz particles. The amount of clay is related to closeness of the deposit to the source. An indirect measure of sensitivity is given by Skempton's⁶ "activity" which for bluff-line loess averages ~ 0.46 , clearly in the "inactive" range.

The inactive particle theory seems to have significance to loess stability, however, to investigate the theory fully thermogravimetry will be necessary to

quantify the amount of active minerals present in each of the various particle-size fractions.

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SMALLEY REPLIES—LUTENEGGER makes quite a good case for considering loess a sensitive soil, and for applying the inactive-particle short-range-bond hypothesis to it, but if we are going to take this approach we shall have to acknowledge the pioneering opinions of Denisov¹. He stated that a subsident loess (the sort described by Lutenegeger) is extremely sensitive, particularly when saturated. The water content in such a state is above the liquid limit; the condition which occurs in the classic quickclays. Denisov pointed out that loess in this state preserves some strength and can form steep sides to canals and pits. When disturbed the shear strength drops to zero. The basic reason, according to Denisov, for the appearance of highly sensitive quickclays is their "preservation under natural conditions of the uncompressed state": an observation which could be very close to the truth.

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Matters Arising

Matters Arising is meant as a vehicle for comment and discussion about papers that appear in *Nature*. The originator of a Matters Arising contribution should initially send his manuscript to the author of the original paper and both parties should, wherever possible, agree on what is to be submitted. Neither contribution nor reply (if one is necessary) should be longer than 300 words and the briefest of replies, to the effect that a point is taken, should be considered.

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BOOK REVIEWS

Sowing the seeds of an empire

A. Hunter Dupree

FOR a century, from 1760 to 1860, the dominant pattern for a scientific career little resembled the academic progression of the twentieth century. A taste for genteel collecting and an irrelevant university or almost irrelevant medical education was the total preparation for youths of scarcely twenty to embark on an epic journey or ocean voyage. By the time they were homeward bound, the still-young men were not only rich in experience but splendidly and broadly educated scientists, capable of thinking in global terms. Typically, an intense period of working on the collections of the voyage led to a geographically static later life. Darwin's career is an extreme case of this pattern, since he never left Britain after the return of the *Beagle* in 1836 at the age of twenty-seven. A decade later, T.H. Huxley used the voyage of the *Rattlesnake* for his scientific and worldly education. The prototype of this career pattern was Joseph Banks's circumnavigation of the world between 1768 and 1771, as the botanist on that prototype of the scientific exploring expedition, Captain James Cook's first voyage to the Pacific.

Banks, descended from a well-to-do Lincolnshire family, was an indifferent scholar at Harrow, Eton and Christchurch, Oxford, but his interest in natural history led him in 1766, when he was only twenty-three, to go to Newfoundland and Labrador aboard a naval vessel. A.M. Lysaght's *Joseph Banks in Newfoundland and Labrador, 1766* (Faber & Faber: 1971), is a beautiful and definitive book, with an analysis of the natural history and a collection of diaries and documents.

The young Banks paid his own way, and that of his illustrators and collectors, as botanist on Cook's expedition to observe the transit of Venus at Tahiti in 1769. A pattern of specialization, both among scientific disciplines and between naval officers and civilian scientists, was already well established. Cook the hydrographer and geographical explorer would have accomplished less without the civilians, and an astronomer alone could not have brought home the contributions to botany, zoology and anthropology which Banks's personal skill and financial resources made possible. Thanks to *The Endeavour Journal of Joseph Banks 1768-1771*, edited by J.C. Beaglehole (Angus & Robertson: 1962), Banks's account of the voyage has been available to scholars around the world, bringing to bear in extensive notes the scholarship of many experts, including Averil Lysaght.

Sir Joseph Banks: 18th Century Explorer, Botanist and Entrepreneur. By Charles Lyte. ISBN 0-7153-7884-8. Pp.248. (David and Charles: 1980.) £10.50, \$32. *The Journal of Joseph Banks in the Endeavour 1768-1771*. With commentary by A.M. Lysaght. Facsimile edition, 2 vols, limited to 500 copies. Vol.I, pp.616, ISBN 0-904351-03-3; Vol. II, pp.732, ISBN 0-904351-04-1. (Genesis Publications, Guildford, Surrey: 1980.) £230.

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REASONS

Joseph Banks, detail of a portrait by Francis Cotes. The painting was only recently rediscovered and is reproduced for the first time in the facsimile edition of the Endeavour journal. Executed in the middle 1760s, the portrait is of interest as the only pictorial record of Banks before he embarked in the Endeavour.

Banks's enthusiasm for the role which he had assumed on Cook's first voyage made his plan for the second so expansive that he came into conflict with Cook and the Navy Board, and the sailing qualities of the ship he sought to modify added further problems. When the superiority of military command to scientific command was decisively confirmed, Banks and his entourage stayed at home.

He assuaged his injured pride by a brief private expedition to Iceland in 1772, returning to England to remain almost without exception until his death in 1820. A friend of King George III, he became adviser to the King on the development of the Royal Botanical Garden at Kew and informally its permanent director, and President of the Royal Society of London in 1779. In a review of Beaglehole's edition of the *Endeavour* journal in the *Times Literary Supplement* (July 20, 1962, p.523), the reviewer said:

He [Banks] was to become a sort of scientific adviser at large, a central clearing house of other naturalists' collections and ideas, perhaps, indeed, the first of the scientific civil servants; and in any case, in his long reign as President of the Royal Society, the exact opposite of the perpetual global tourist for which he had first cast himself. These extended views of his long career need much more careful sorting out.

That call for a well-rounded biography doing justice to science policy was well taken in 1962 and remains unfulfilled today.

The emphasis of Lyte's new biography seriously detracts from its usefulness. Seventy per cent of the book brings one to November 1772, when Banks at the ripe age of twenty-nine returned from his Iceland expedition. Hence scholars can without loss continue to turn to Beaglehole and Lysaght, or even to the previous biographies by H.C. Cameron (1952) and Edward Smith (1911). Lyte does not delve below the surface in his analysis either of the natural history or the science policy that makes Banks such a significant figure.

In spite of this criticism of the scholarly contribution of Lyte's book, one can point out both that it reads pleasantly and that the selection of episodes for emphasis presents an implicit interpretation of some interest. Banks, by this reading, became Cook's negotiator with the peoples on the shore. His anthropological research had the very practical purpose of gaining supplies for the ship and access for landing. The shore parties could then proceed with science, trade and, if possible, sex. The sequence of negotiations often involved the use of firearms by the British, and the show of force frequently produced casualties. Banks then awaited a second approach from the natives which would allow him to move confidently among them, trading nails for supplies, becoming adept in the language and quickly identifying the important people with whom to deal. All of

Courtesy Mr David Hughes. Photograph by John Bignell.

the negotiation was preliminary to vigorous collecting of cultural artifacts, animals and especially plants, including horticultural varieties and weeds — a valuable record for students of the worldwide diffusion of organisms associated with man. Hence natural history became fused with the national and military goals of the expedition, and Banks the botanist became second only to Cook in shaping the policy of European penetration into the southern Pacific — New Zealand and Australia as well as the Polynesian islands, of which Tahiti was the most important. An element in this policy was persuading a likely native or two to continue with the ship to serve as interpreter and guide. The links between scientific, military and national interests, represented by useful plants and animals, became a part of the system by which Britain replaced her lost American colonies with a world empire.

The Journal of Joseph Banks in the Endeavour, with a commentary by A.M. Lysaght, is a facsimile of the original manuscript in the Mitchell Library in Sydney, Australia. The commentary breaks new ground with reflections on Banks as an anthropologist. The extensive index takes full account of Banks's lapses in pagination and provides firm control of both scientific and geographical names. Only the most specialized of scholars will have occasion to consult in detail the facsimile itself; there is, however, a more important justification for this expensive, limited edition with its fine binding and gilded pages. Not only were the Banks manuscripts withdrawn from the British Museum in 1886 and scattered all over the world by an auction at Sotheby's. Banks himself placed a burden on posterity by allowing the illustrations of the *Endeavour* voyage by Sydney Parkinson and others to go unpublished, even though the copper plates and the descriptions by D.C. Solander were ready by the time of Solander's death in 1782. An important function of the rare book library is to bring these fugitives to light. An alternative way of financing the publication of residues of scientific literature left behind in enclaves for two centuries is hard to conceive, unless unusual beauty or interest attracts the bibliophile. These volumes fit neatly between *Captain Cook's Florilegium*, published by Lion and Unicorn in 1973, and the *Banks' Florilegium* recently announced by Alecto Historical Editions, which at long last reproduces in full colour the engravings of the drawings by Sydney Parkinson. The facsimile edition contains an extensive list of the illustrations. Although Banks the President of the Royal Society has yet to find his biographer, the Banks of Cook's first voyage is now increasingly well-served by the literature. □

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Princeton's obeisance to Einstein

Paul Davies

Some Strangeness in the Proportion: A Centennial Symposium to Celebrate the Achievements of Albert Einstein. Edited by H. Woolf. Pp.538. ISBN 0-201-09924-1. (Addison-Wesley: 1980.) \$43.50, £26.10.

EINSTEIN'S conception of the physical world was so broad, it is appropriate that a centennial symposium should deliberate on topics as diverse as quarks and quasars. The hundredth anniversary of the great man's birth was marked by a variety of international gatherings and the publication of several books. These activities ranged in subject across Einstein's political and religious convictions, his contributions to statistical mechanics, relativity and quantum theory, his philosophical deliberations and his cosmological speculations. Princeton's symposium had some of each.

Science in general, and physics in particular, is often presented as an impersonal, logical tide of progress, rooted in narrowly defined procedures of experimental test and mathematical precision. The reality is different. Like so many human activities, the development of physics revolves around personalities, with their attendant prejudices, beliefs and jealousies. Although ultimately resting on empirical foundations, the actual cut and thrust of mainstream research is dictated by individuals and mirrors thus their tastes and aspirations. This individualism is reflected in the scientific community's creation of idols. Great physicists — Newton, Laplace, Maxwell, Fermi, etc. — are exalted, almost deified by succeeding generations of their profession. Einstein is one of the few scientists whose awesome genius attracted the fascinated adulation of a much wider public: the Gandhi or Napoleon of physics.

The impact of Einstein's theoretical work on the subsequent development of physics is legendary. At a stroke he helped initiate two revolutionary new conceptual structures — special relativity and the quantum theory — and completed one of them (his general theory of relativity) so decisively that he left his colleagues with little more fundamental work to do on the subject for the next 40 years. Because these theories, mostly presented between 1905 and 1915, later assumed such immense importance for physics, almost every facet of modern research incorporates Einstein's ideas somehow. Nevertheless, science moves on. One detected, perhaps, a somewhat strained quality in the universal claims of the participants that their particular branch of galactic dynamics or gauge theory or particle physics is but a natural and inevitable extension of the patriarch's great intellectual empire. What would Einstein really have made of gluons or gravitinos?

How does one celebrate Einstein's achievements? Scientifically the formula is universal: astrophysics and particle physics, with special emphasis on their intersection. Undoubtedly the event of the centennial epoch was the discovery of gravitational radiation, predicted by Einstein himself over 60 years ago, and persuasively confirmed by the recent observations of the so-called binary pulsar. On the theoretical front the swift progress towards unifying the four fundamental forces of nature dominates the scene. At the Princeton symposium the gravitational component of this confluence — supergravity — provided a natural complement to the many experimental papers.

In spite of these specialist details, the perspective was all-embracing. "The Universe" was reviewed and analysed, its mysteries and paradoxes set alongside its simplicity and splendour. Where did the cosmic material come from, where is the antimatter, why is the expansion so uniform, what happened in the beginning, how will it end . . . ? The contributors were all experts of international renown, and between them they covered all the puzzles, problems, successes and failures of modern cosmology.

Special relativity was not submerged by the general theory. Wolfgang Panofsky explained how the principles of the special theory are these days a matter for routine engineering work, in such devices as high-energy klystrons and accelerators. I suspected a side-swipe at the malcontents who still insist, all these years on, that there is something fishy about time dilation, length contraction and all the other weird effects predicted by Einstein's theory. Most memorable of Panofsky's comments is that to a high-speed electron, Stanford's two-mile linear accelerator seems only about 30 inches from end to end!

For many readers the more attractive part of these symposium proceedings will relate to the papers about Einstein himself, and those heady days in the first quarter of this century when science was turned upside down and major advances jostled each other in the literature. For today's physicists, groping among the complexities of a tangled and increasingly fragmented subject, the anecdotal images of a serene intellect nimbly picking its way through half-a-dozen major scientific conundrums makes compelling reading. Of course, he was not always right. His implacable opposition to the claims of Bohr, Born and others that quantum theory was a complete description of nature made him a curiously isolated figure — "like an ostrich", he himself remarked, unable to face the "evil quanta".

"I knew Einstein", in spite of its I-danced-with-the-Prince-of-Wales overtones, is surely an impressive and riveting

revelation. Several of the symposium participants either knew, or at least spoke to this Swiss-German-American Jew. No narrow-minded scientist he, we are assured. Einstein liked (even played!) chamber music, discussed Spinoza, rode on trams. He also lived through a time of intense political and social upheaval, and championed a variety of causes.

Remembering Einstein evokes among physicists a curious amalgam of humility and conceit. Who could ever aspire to such a complete mastery over their chosen subject? Measured against the Einstein standard, we are all lesser scientists. Yet we, the community of physicists, claim Einstein as one of our own, and love to dazzle each other, and the wider public, with the brilliant advances that Einstein stimulated.

Was Einstein a true genius who would have succeeded in any chosen activity, or

was he merely a man of the moment, thinking in the right way at the right time about the right sort of problem? If he lived today, would his contributions to physics be as profound and far-reaching? Somehow I do not think so. Yet that is not to demean his achievements, which are paralleled only by Newton. Because of Einstein the world is a far richer place.

This centennial volume, like the others, is a patchwork. The joins are smoothed somewhat by augmenting the technical papers with comments from subsidiary speakers, together with a few rather unsatisfactory open discussions involving a good deal of rambling. It is, however, a delightful addition to one's library, and an invaluable source of Einstein stories. □

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Limb diversity, what can be more curious?

Julian Lewis

The Development of the Vertebrate Limb: An Approach through Experiment, Genetics, and Evolution. By J.R. Hinchliffe and D.R. Johnson. Pp.266. ISBN 0-1985-7552-1. (Clarendon/Oxford University Press: 1980.) £20, \$59.

THE question of the relationship between evolution and embryonic development is forever tantalizing. The experts on each of these topics feel that somehow they hold the key to the understanding of the other; and yet it is remarkably hard to translate the feeling into a real and useful new insight. Hinchliffe and Johnson preface their monograph with an apt quotation from Darwin:

What can be more curious than that the hand of a man, formed for grasping, that of a mole for digging, the leg of a horse, the paddle of the porpoise, and the wing of the bat, should all be constructed on the same pattern, and should include similar bones, in the same relative positions?

Though limb proportions have varied, limb topology has remained practically constant. This, as Darwin saw, is evidence that structures evolve in a quasi-continuous fashion, by a succession of small adjustments, rather than by big, single leaps that change the qualitative plan. The important implication for the developmental biologist is that even though the quantitative parameters of limb development differ from species to species, the qualitative principles can be expected to remain almost the same. The search for general mechanisms rests on that proposition. But it is still a proposition that needs to be substantiated directly. Thus Hinchliffe and Johnson's book, though it does not offer any new theoretical insights,

is valuable for the comparative information that it provides.

The authors begin with an account of the evolution and of the diverse adaptations of the vertebrate limb. The core of the book is a review of descriptive, comparative and experimental studies of limb development and regeneration. The final chapter discusses limb mutations. The book is well-written, informative and at times even entertaining. It is, however, rather uneven in its coverage, and, like many reviews, it suffers from the lack of a unifying central thesis. I felt this lack particularly in the chapters on development. These focus on the genesis of the limb skeleton, and touch on many other interesting topics, including some fundamental problems of developmental biology; but the account does not seem to be informed by any clear general conception of the principles of development. Perhaps the authors simply wish to be objective, and to keep their prejudices from obtruding. But it is consequently hard to tell which experiments are to be considered crucial, which models important, which theories right. To borrow a phrase from Hinchliffe and Johnson, I fear that the general reader may be left asking: "Amidst this welter of morphological anomaly and theoretical speculation, what conclusions can we draw . . .?"

The declared aim of the authors is to provide "a reference book which is at the same time an account of the current 'state of the art'." As such the book should be useful; the experts will be able to bring their own prejudices to the reading of it.

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Peter Dunnill

Immobilized Enzymes in Analytical and Clinical Chemistry: Fundamentals and Applications. Chemical Analysis, Vol.56. By P.W. Carr and L.D. Bowers. Pp.460. ISBN 0-471-04919-0. (Wiley: 1980.) £31.10, \$63.20. *Immobilized Enzymes: An Introduction and Applications in Biotechnology.* By M.D. Trevan. Pp.138. ISBN 0-471-27826-2. (Wiley: 1980.) £8.75, \$24.10.

THE great development which the field of immobilized enzyme technology has undergone is reflected in quite different ways in these two books. Ten years ago the field was so specialized that there was little need for a simple introductory text, but Michael Trevan's slim volume is published at a time when many people, including teachers, industrialists and even investors, need to know about the implications of enzyme technology, and have some grasp of the underlying principles. Equally, a subject such as the use of immobilized enzymes in analysis, which a decade ago warranted only a review chapter, now not only justifies a book, but Professors Carr and Bowers have had to add supplementary reference lists to deal with the outpouring of recent papers.

Dr Trevan divides his introductory account into three main parts. The first, dealing with the techniques of immobilization and its consequences for enzyme activity, is noteworthy for its clear and readable explanation of immobilized enzyme kinetics and the consequences of diffusional effects. The second reviews the current and potential applications of immobilized enzymes. This survey is comprehensive, but even more lead references would help to avoid any frustration in readers who wish to pursue more detailed information. The final section reflects renewed interest in the relationship between artificially immobilized enzymes and the functioning of enzymes which are fixed spatially within the cell, for example by association with membranes. Though the models which can be constructed to mimic active transport and other cellular processes are as yet crude, they point the way to exciting scientific developments. Introducing the possible industrial consequences of multi-enzyme catalysis to biochemistry students at the earliest possible stage could help to knit further the scientific and technological aspects of the subject.

The monograph by Carr and Bowers is a contribution to a series on analytical chemistry and sets out to provide a self-contained explanation of the use of immobilized enzymes in analytical and clinical chemistry. Therefore it deals with basic enzymology and enzyme kinetics, and with appropriate immobilization techniques. Like Trevan's book, a sub-

stantial part of the volume has to be devoted to the kinetics of immobilized enzymes, since a grasp of this aspect is central to interpretation of analytical results; however, as one would expect, the treatment is of greater depth and rigour, and this extends to the discussion of individual analytical methods where the focus is rightly on the underlying principles. The authors accept that immobilized systems have not made as much headway as expected, given the potential benefits in terms of reduced costs and increased accuracy. They ascribe this in part to the additional complexity of the two-phase enzyme-reagent system and to the need for a new method to show

substantial advantages in order to displace established practice: anyone who has observed the pressures on hospital analytical facilities will know that introducing untried innovations is unlikely to be popular. However, the book's discussion of the strengths and weaknesses of immobilized enzymes for analysis should provide a firm foundation for the development of commercial systems.

Both books succeed in achieving their very different objectives and are valuable additions to the growing library of enzyme technology literature. □

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Pollution assessment: objectives and means

F. Moriarty

Biological Monitoring for Environmental Effects. Edited by Douglas L. Worf. Pp.225. ISBN 0-669-03306-5. (Lexington Books: 1980.) £15.50, \$22.95.

THIS is the report of a conference and workshop held in North Carolina early in 1978. It was an all-American affair, and there is a lot of detail in the 22 papers that will not be of much interest to readers from other countries. Nevertheless, this book does raise several themes of general relevance for those who are concerned with monitoring the environmental effects of pollutants.

Biological monitoring was taken to mean the use of biota both to monitor the structure and function of ecosystems, and to measure the amount and distribution of contaminants. Several participants were concerned that current and impending regulations rely solely on chemical and physical measurements: the possibilities of, and need for, biological monitoring are not appreciated. Cairns and Dickson both emphasized that control of pollution by the "best applicable technology" is inappropriate, because it ignores the environment's capacity to deal with waste materials, and can result, in different circumstances, in either too much or too little protection of the environment.

A linked theme is the question of objectives. An adequate monitoring scheme cannot be designed until precise objectives have been stated. Weber discusses one aim of the Federal Water Pollution Control Act, to restore and maintain the biological integrity of the nation's waters. Biological integrity is not explicitly defined, and unless it means that ecosystems should remain in, or be restored to, their pristine condition, it is a

nebulous concept that does not suggest objectives for monitoring. In practice, it is taken to mean fish, shellfish, wildlife and the effects of pollutants on the diversity, productivity and stability of communities of indigenous aquatic organisms. Osburn refers to the National Environmental Policy Act, which clearly accepts values such as aesthetic and cultural pleasure, and which can be translated into clear objectives.

Hirsch emphasizes that human impact on wildlife is synonymous with effects on ecosystems, discusses the scientific problems and then lists three institutional difficulties: lack of assured long-term funds for relevant ecological research, inadequate communication between administrators and ecologists, and lack of a focal point for expertise on ecological problems.

Other papers describe a range of techniques for monitoring in aquatic and terrestrial habitats, and the last five summarize the conclusions of five workshops. Current research is criticized for producing too many data of uncertain relevance; in addition more taxonomic skills are needed and a better ability to communicate scientific data to administrators. Research is also hampered by academic and administrative arrangements that militate against the development of multidisciplinary teams.

Several recent books have dealt with various aspects of monitoring. The range of topics in this book is limited, and much of the detail is of relatively parochial interest, but the problems encountered have a much wider relevance.

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Provocative images through the microscope

P. W. Hawkes

Scanned Image Microscopy. Edited by Eric A. Ash. Pp.461. ISBN 0-12-065180-7. (Academic: 1981.) £18.40, \$49.

So accustomed are we to all the conveniences of two-dimensional image formation at optical wavelengths — the existence of lenses and of high-resolution polychromatic recording media in particular — that it is easy to forget that information can often be usefully conveyed by other fields for which no such facilities exist. The absence of lenses for X-rays, at least until very recently, is an obvious example. In such cases, the information may well be useful enough for other means of collecting it to be developed, and this is one of the reasons why the scanning principle is used. Its most obvious advantage arises when the incident radiation can be concentrated into a small spot on the specimen but cannot be focused after interacting with the latter, so that there can be no question of conjugate planes: acoustic microscopy is an example of this. Here, the specimen is scanned mechanically under the spot and a signal collected for each position.

Scanning is thus used for similar reasons in quite different domains, and this volume of Rank Fund Conference Proceedings contains accounts of four types of scanning microscopy: acoustic, optical, photoacoustic and soft X-ray microscopy. Scanning electron microscopy is excluded, on the grounds that it is now too well-known, which is true of the traditional reflection instrument but not of its much newer and still rather uncommon transmission counterpart. It is a pity that some contributions on the latter were not solicited, for the resemblances and differences between the scanning transmission electron microscope and the scanning optical instrument are interesting and this would have been an excellent opportunity to compare and contrast.

Even with this minor cavil, however, the coverage is thorough. Each of the four main sections contains chapters by the inventors or leading exponents of the instrument in question. Thus acoustic microscopy is discussed by C. F. Quate, H. K. Wickramasinghe and B. Nongaillard, as well as by several other contributors concerned with specific applications. Scanning optical microscopy is examined by W. T. Welford, G. J. Brakenhoff and colleagues, and by T. Wilson and C. J. R. Sheppard, among others. The other two sections have no less distinguished a cast. The result is that although the book has all the faults inherent in such proceedings' volumes — much repetition in the chapter openings in particular — it does provide a clear and readable introduction to these four scanning-based types of microscopy and gives a good idea of their potential. I

suspect, too, that practitioners of each technique will find it an extremely useful guide to what is happening in related fields, particularly when these are used to complement one another (the example of scanning optical and electron microscopy, cited in the optical section, is one of many).

In short, this is an extremely useful collection: the individual contributions are long enough for the newcomer to be able to follow and the choice of scanning as leitmotiv makes good physical sense. Apart from the omission mentioned above, the absence of one other topic is to be regretted: spectroscopy using orthogonal transforms. Here, it is the need to "scan" a field with small detectors that has provided the incentive to seek new solutions, resulting in Hadamard transform techniques for example. Nevertheless, it is probably because the material that E. A. Ash has assembled for us is up to date, well presented and frequently provocative that we should have liked a little more.

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Pelagic predators

Geoffrey Fryer

Predation and Freshwater Communities. By Thomas M. Zaret. Pp.187. ISBN 0-300-02349-9. (Yale University Press: 1981.) £9.50, \$15.

THIS book considers predation on the basis of evidence obtained in the wild, a welcome change from the generation of endless equations. The title notwithstanding, it is largely confined to freshwater zooplankton communities. Here, changes in the level and kind of predation can have dramatic effects on species composition and may even lead to complete restructuring. It is to these phenomena that Zaret addresses himself. Unfortunately the outcome is disappointing.

Two types of predators, gape-limited and size-dependent, are recognized. These, in effect, are vertebrates and invertebrates respectively. The logic of referring to fishes, much the most important vertebrate predators on the zooplankton, as gape-limited in this context is puzzling, for it is admitted that gape limitation is at most "of very brief duration in the fish's life and can be ignored for most considerations". To regard hemipterous insects, the only invertebrates so classified, as gape-limited seems equally odd.

The ways in which animals react to increased predation or pressure from different kinds of predators are described.

CHROMOSOMES TODAY

VOLUME 7

EDITED BY

M. D. BENNETT
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George Allen & Unwin announce the publication of the proceedings of the Seventh International Chromosome Conference held at Oxford in 1980. The volume provides up-to-date short reviews of a wide range of chromosome topics by leading authorities. It will be invaluable to all hard-pressed biologists who wish to keep abreast of recent research in the field. The main subject areas covered are: chromosome organisation at molecular level; chromosomes in meiosis; population cytogenetics; chromosomes and speciation; chromosomes and plant breeding; chromosomes and malignant change; induced chromosome variation and human cytogenetics.

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Small size or inconspicuousness achieved by various means enables some planktonic animals to evade fishes, and the possibility of doing so by vertical migration is also considered. Invertebrate predators can sometimes be thwarted by other means, as by protective spines or shapes that are difficult to handle, by escape reactions or simply by large size. Complexities arise when both types of predator are active: an anti-fish stratagem may increase vulnerability to a copepod. On the basis of case histories, somewhat subjective models are described which predict reactions to various kinds of predation and are shown to be in reasonable agreement with certain real life situations. Nature is, however, almost infinitely diverse and some categorical statements are incorrect. Thus *Bosmina* is not "dominant only when gape-limited predation is intense" — I know a score of fishless water-bodies where *Bosmina* dominates the open water, often alone — and it is nonsense to say that *Daphnia magna* (not truly planktonic) and *D. pulex* cannot co-exist. My records reveal *D. pulex* as the most frequent conspecific associate of *D. magna*.

And so to the factual errors, of which there are so many one hardly knows where to begin. The author does not know much about copepods or he would not refer to "large conspecifics" — adults do not moult — or ask "what function is served by their compound eyes?", organs they do not possess. Cyclopoids do not stab prey from behind with daggerlike mandibles. No mere quibble this: to understand protective

devices we must know how the predator attacks. The statement that copepods "will attack neither very large nor very small prey" is also erroneous; cyclopoids that attack animals much larger than themselves, including small fishes, can be reared on protozoans. Considering its importance in the theories developed, size is indeed treated very carelessly. Some species of *Ceriodaphnia* attain more than twice the cited maximum size; the grotesque full-page figure of *Holopedium* bears the wrong scale; and to suit a model three daphnids are lumped together "because they are within a range of 1.0 mm", forgetting that in these small animals this reflects a difference of 125 per cent.

Other animals are equally abused. The full page figure that purports to be a *Chaoborus* larva (indexed as the phantom midge!) is only a head with part of the gut attached; the figure labelled *Bythotrephes longimanus* is *Polyphemus pediculus*; cladocerans not rotifers produce ephippia; stomatopods are not pistol shrimps nor notonectids water striders, and so one could go on. Carelessness, including a host of mis-spelled names, sometimes provides amusement — otter densities of 20 to 30 per m² must be as remarkable a sight as fishes "feeding on the mollusc's feet".

Revised and re-written this could be a useful little book: as it stands it cannot be recommended. □

Geoffrey Fryer is a Senior Principal Scientific officer at the Windermere Laboratory of the Freshwater Biological Association.

Isotopes and geology

Gunter Faure

Handbook of Environmental Isotope Geochemistry. Vol.1, *The Terrestrial Environment*, A. Edited by P. Fritz and J.Ch. Fontes. Pp.532. ISBN 0-444-41780-X. (Elsevier Scientific: 1980.) Dfl.185, \$90.25.

THIS is the first of a proposed set of five volumes dealing with applications of isotopic studies to a wide variety of problems, ranging from the geochemistry of forest soil to igneous petrology. The first volume on the terrestrial environment gets off to a shaky start with a preface and an introduction by the series editors. They justify their ambitious undertaking with the claim that isotope geology reached its present importance in the earth sciences only in the context of geochemistry, hydrogeology and environmental geology. Applications of isotopic studies to dating of terrestrial (or extra-terrestrial?) materials and to palaeoclimatic studies are judged to be of limited practical importance; this point of view may explain why isotopic studies of interest "to economic and hardrock geologists" are relegated to the fifth volume. Yet, in spite of several factual errors, the introduction does contain much useful information on the systematics of isotope fractionation that may assist the reader in understanding technical details of the following twelve, more substantive, contributions.

The first five chapters are devoted to presentations of isotope fractionation in different terrestrial environments including the atmosphere, groundwater, ice and snow, and geothermal systems. These are followed by seven chapters dealing with more specific topics. All of the contributions were written by experts in their respective fields and contain a wealth of detailed information regarding isotopic and geochemical processes in different terrestrial environments. The articles by Deines on reduced carbon, Osmond on uranium disequilibrium in ground water, Savin on mineral-water interactions and Krouse on sulphur compounds in the environment are especially well organized and concisely written. Several others are a bit of a chore to read because they are long and discursive.

This book will be useful primarily to scientists in search of information about isotopic and geochemical studies when their own research impinges on the natural processes occurring on the Earth's surface. Each chapter is attended by a lengthy list of references that range over several decades from the early 1950s up to and including 1978, and there are detailed author and subject indexes which enhance the value of the book as a source of information. □

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Physics and chemistry of membranes

Ora Kedem

Basic Principles of Membrane Transport. By S.G. Schultz. Pp.204 Hbk ISBN 0-521-22992-8; pbk ISBN 0-521-29762-1. (Cambridge University Press: 1980.) Hbk £14, \$22.50; pbk £4.95, \$8.50.

ALTHOUGH various treatises dealing with general aspects of membrane transport or membrane function are available, this book is a valuable addition to the literature. It is a unified presentation, starting from first principles and including discussions of some of the more important transport systems in the living organism. Nevertheless, it is appropriate that the title does not include any "bio-" key word because the author provides the physico-chemical foundation for studying biological membranes, and does not attempt a summary of the detailed and rapidly growing physiological and biochemical information.

The structure of the book is logical and not historical; thus, for example, the GHK flux equation is thoroughly discussed in the chapter on isothermal diffusion, where it belongs. In the chapter on some principles

of electrophysiology, the concept can then be applied in context. The brief summaries, at the end of some chapters, state any conclusions that one can draw from the relatively simple treatment and stress their limitations. Indeed, a feature of the book as a whole is that assumptions and arguments are clearly stated. While a formal quantitative account is given in enough detail to be readily followed by the reader, emphasis is on the physical significance of the results, and in many cases the physical meaning of steps in the derivation.

With this recommendation to both students and researchers in the field, a note of reservation is necessary. The desire for intuitively appealing explanation has led in some cases to inaccurate, or at least non-rigorous, statements. However, considering the logical development of the book as a whole — and the detailed references — these should not lead to any serious misunderstandings. □

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